Delineating the Role of Stress Granules in Senescent Cells Exposed to External Assaults

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Abstract

As we age, our ability to cope with a variety of stresses significantly decreases. One of the features of an ageing organism is the dramatic increase in the number of cells arrested in the G1 phase, a process known as senescence. It is well established that the senescence phenotype leads to a change in the way cells respond to stress. However, the molecular mechanisms by which these cells cope and/or respond to a variety of environmental challenges remain unknown. In general, cells respond to stress by engaging a variety of mechanisms; one of them is the assembly of cytoplasmic foci known as stress granules (SGs). These entities are considered as part of the survival pathways that are activated at the beginning of any stress to protect key cellular elements which allow a quick recovery if the stress is rapidly removed. However, we do not know whether SGs formation is activated during senescence. In this study, we investigated the formation and the role of SGs in senescent cells exposed to various stresses. We demonstrated that while SGs can assemble in response to oxidative stress (OS) during all the steps leading to senescence activation, their number significantly increases at late stage of senescence. This increase correlates with a rapid decrease in the expression of the cyclin kinase inhibitor p21, one of the main players in the activation of the senescence phenotype. Although the OS-induced recruitment of p21 mRNA to SGs correlates with a significant increase in its half-life, this translocation interferes with p21 translation only at late senescence. This translation inhibition could be explained by the co-recruitment of CUGBP1, a known translation activator during senescence of p21, and p21 mRNA to SGs. Therefore, our data suggest that SGs formation and the reduction in p21 protein levels represent two main events through which senescent cells respond to stress conditions.

Résumé

Les cellules sénescentes sont plus sujettes à des contraintes que les cellules prolifératives. Les cellules prolifératives répondent à des contraintes en formant des granules de stress, mais aucune publication n'est disponible sur l'induction des granules de stress dans les cellules sénescentes. Dans cette étude, nous avons étudié l'induction des granules de stress dans les cellules sénescent sous différentes contraintes. Nous avons également investigué sur la réponse des cellules sénescentes à l'induction des granules de stress. Nous avons constaté que les granules de stress peuvent être induites dans les cellules sénescentes, et que le nombre de granules de stress augmente pendant la sénescence. L'induction des granules de stress peut entraîner un décalage de la voie de phosphorylation dépendante de eIF2α dans les cellules prolifératives à la voie de phosphorylation indépendante de eIF2α dans les cellules sénescentes. Nous avons aussi constaté que le message de p21 est recruté dans les granules de stress et est stabilisé dans la sénescence. Et pour la première fois, nous avons constaté que la protéine de liaison à l'ARN CUGBP1 est recrutée dans les granulés de stress pendant la sénescence. Le recrutement du message de p21 et la protéine CUGBP1 dans les granules de stress mène à une diminution significative de la protéine p21 sous l'effet du stress dans les cellules sénescentes. Enfin, nous montrons que les cellules sénescentes récupérent plus lentement que les jeunes cellules en terme de démontage de granules de stress et de l'expression de la protéine Hsp70.

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Introduction

As young cells become progressively old, many changes such as altered gene expression, accumulation of damaged proteins and impaired proteasome activity occur. Therefore, several biochemical and phenotypical differences exist between old and young cells. One pronounced characteristic of old cells is their declined adaptive capacity to environmental stresses (Helenius et al., 1999; Bruunsgaard and Pedersen, 2000). It is well known that as they age, many cells in our organism enter irreversible cell cycle arrest, a phenotype known as senescence. Recently, increasing evidence has shown that cellular senescence may contribute to ageing (Krtolica and Campisi, 2002). Furthermore, senescent cells may also contribute to the development of cancer in aged individuals due to their ability to modify the surrounding microenvironment. It has been shown that senescent human fibroblasts can stimulate pre-malignant and malignant proliferation of epithelial cells and tumor formation in mice by excreting extracellular factors (Krtolica et al., 2001). Moreover, senescent cells may contribute to other age-related diseases such as atherosclerosis (Krtolica and Campisi, 2002). Therefore, understanding the process through which cells undergo senescence and how these cells react to stress can help us understand the ageing process and combat late-life related cancers and other diseases. In this study, we investigate whether stress granules, cellular foci that form upon cell's exposure to a variety of stresses, can be formed in senescent cells under conditions of oxidative stress as well as their functional relevance in the mechanisms that help these cells react to environmental assaults.

Cellular Senescence

Since the initial discovery of cellular senescence (Hayflick, 1965), many researchers have been involved in discerning the mechanisms that modulate this process. Normal human diploid fibroblasts have a limited proliferation capacity and after a finite number of population doubling, they enter irreversible growth arrest known as replicative senescence. Although in senescent cells, processes such as gene expression and translation are still functional, these cells do not respond to physiological mitogens (Krtolica and Campisi, 2002). It has been hypothesized that the decreased sensitivity of senescent fibroblasts to a variety of environmental stimulus including mitogens is due to defects in the signaling pathways which mediate the response to these factors (Cristofalo et al., 2004). This hypothesis is supported by the observation that senescent fibroblasts show one more round of DNA replication when infected with Simian Virus-40 (Cristofalo et al., 2004).Therefore, the replicative machinery of senescent fibroblasts is still intact. Senescent cells are morphologically larger and flatter than young cells due in part to an increase in the size of the nucleus and nucleoli (Cristofalo et al., 2004). The main cause of replicative senescence is progressive telomere shortening (Krtolica and Campisi, 2002). However, other recent advances in the ageing field have shown that other factors can contribute to the senescence phenotype such as DNA damage, oxidative stress, overexpression of oncogenes and chromatin structure remodeling (Collado et al., 2007; Marcotte et al., 2004).

I- Stimulators of senescence

I.A.-Telomere shortening

The specialized DNA structure at the end of eukaryotic chromosomes, called telomeres, stabilizes the chromosome ends and prevents chromosomal abnormalities (Wright and Shay, 2000). Telomeres are maintained by a specialized enzyme named telomerase which is not expressed in most normal human somatic cells (Nakayama et al., 1998). Human telomeres are programmed to shorten by about 100 base pairs after each population doubling. Human cells enter senescence after an average of 50 population doublings (Karlseder et al., 2002). When the telomeres are shorter than a critical length, the DNA damage response signals are initiated and the p53-dependent checkpoints are activated, leading to cellular senescence (Smogorzewska and de Lange, 2002). Telomere length is thought to function as a mitotic clock that counts the number of cell divisions of human normal somatic cells (Ohtani et al., 2004). Therefore, telomere-dependent mechanisms are involved in cellular senescence in human cells. Overexpression of the telomerase, hTERT, in cultured cells is sufficient to prevent telomere shortening resulting in the immortalization of human fibroblasts (Vaziri and Benchimol, 1998). Furthermore, the importance of telomere in this phenotype is also confirmed by the observation that mice deficient in telomerase activity have short telomeres and undergo premature ageing (Blasco et al., 1997) and the liver of these hTERT-/- mice presents an increased number of senescent hepatocytes (Satyanarayana et al., 2003). Thus, there appears to be an inverse correlation between the length of telomeres and the onset of senescence in different tissues (Ogami et al., 2004). Another study has shown that the rate of telomere shortening is accelerated under conditions of oxidative damage (which accumulates with aging) (von Zglinicki and Martin-Ruiz, 2005). Therefore, telomere shortening in addition to reflecting the proliferative history of cells also indicates the degree of oxidative damage (Collado et al., 2007; d'Adda di Fagagna et al., 2003). Dysfunctional telomeres

can be visualized in the cell as foci containing the phosphorylated histone γ-H2AX which colocalizes with telomere providing a marker for telomere shortening (d'Adda di Fagagna et al., 2003).

The molecular mechanism mediating telomere-loss-induced-senescence is thought to involve the protein ATM and its targets Chk1 and Chk2 which are important for the activation of the p53 pathway (Ben-Porath and Weinberg, 2005). A recent study has shown that the cyclin kinase inhibitor p21, a downstream target of p53, is relevant in the telomeres shortening in mice (Choudhury et al, 2007). Deletion of the p21 gene extends the lifespan and improves the general fitness of telomerase-deficient mice. Another protein involved in the shortening of telomeres is the component of the DNA mismatch repair machinery, the DNA repair factor PMS2 (Siegl-Cachedenier et al., 2007). PMS2 triggers proliferation arrest and ageing by telomere shortening in a process that appears to be upstream of p21.

Although telomere shortening is considered the prominent cause of telomere-driven senescence, one study has shown that it is not the telomere loss but rather the state of the telomere that is involved in cellular senescence (Karlseder et al., 2002). In this study the authors show that the overexpression of TRF2, a telomeric DNA binding protein, increases the rate of telomere loss without accelerating senescence. TRF2 protects critically short telomeres from fusion, represses chromosome-end fusions and thus delays senescence.

It is assumed that telomere-regulated ageing could be the consequence of telomere shortening in stem cell pools which leads to tissues with high rate of senescence and a progressive exhaustion of the regenerative potential of stem cells (Collado et al., 2007).

I.B.- Oxidative stress

The induction of cellular senescence by oxidative stress is well documented. Although molecular oxygen is very important in cellular respiration and for aerobic life, it can also be toxic if used at the wrong concentration (Cristofalo et al., 2004). Oxygen toxicity results from changes in the rate of generation of ROS (Reactive Oxygen Species) in the cell. ROS is the byproduct of cellular metabolic pathways, including oxygen-centered free radicals. Free radicals produced in metabolic pathways are assumed to constantly bombard cellular components while increasing entropy and decreasing the functional capacity of the cell (Harman, 1956), thus resulting in the induction of senescence.

Cultured cells are maintained at an atmospheric concentration of oxygen (20%) which is much higher than that observed in most tissue. The growth of adult skin cells has been shown to be considerably decreased under normal atmospheric concentration of oxygen when compared with those under a lower concentration of oxygen (5%) (Balin and Pratt, 2002). When human fibroblasts are grown under high oxygen concentrations (40-50%), they undergo premature senescence; in contrast, when these cells are grown under low concentration of oxygen (2-3%), their proliferative lifespan is extended (Packer and Feuhr, 1977). Exposure of cells to oxidative stress by treating them with hydrogen peroxide (H_2O_2) can also induce senescence due to the increased damage of DNA. H_2O_2 treatment of human diploid fibroblasts leads to damaged DNA which affects the ability of

these cells to respond to growth factors (Chen and Ames, 1994). Treatment of cells with H_2O_2 also induces cellular senescence by increasing the production of ROS. (Blander et al., 2003). Rodent cells cultured under 3% oxygen do not show a clear senescent phenotype due to their high rate of spontaneous transformation into an immortalized phenotype. However, when those cells are cultured under a high concentration of oxygen, (20%) they become senescent before they develop into immortalized colonies (Parrinello et al., 2003). Under these conditions, elevated oxygen levels increase DNA damage resulting in the induction of senescence in a p53 dependent manner.

Oxidative stress may affect telomere length. Under oxidative stress, the rate of telomere shortening in human fibroblast cells is accelerated, which leads to the shorter proliferative lifespan of the cells (von Zglinicki et al., 1995). Another study has shown that the lifespan of rat aortic vascular smooth muscle cells is extended when these cells are cultured under hypoxic condition (1% oxygen). This extended lifespan is believed to result from the phosphorylation of the telomerase catalytic component (TERT) which stimulates the telomerase activity (Minamino et al., 2001).

Although the p53 pathway is shown to be involved in oxidative stress induced senescence, the CDK (cyclin-dependent kinase) inhibitor p16 can also be activated in response to oxidative stress (Iwasa and Ishikawa, 2003). p16 inhibits CDK4 and CDK6, thereby keeping the retinoblastoma protein (pRB) in a hypophosphorylated state and arresting cells in the G1 phase of the division cycle (Shapiro et al., 1998; Shapiro et al., 2000; Shapiro et al., 1995). Loss or inactivation of the *INK4a/ARF* locus (harboring *p16* and *ARF* genes) are among the most frequent alterations seen in human cancers, underscoring the widely recognized role of p16 as a tumor suppressor (Serrano and

Blasco, 2001; Serrano et al., 1996; Serrano et al., 1997). In agreement with earlier observations that p16 levels increased with aging (Nielsen et al., 1999; Zindy et al., 1997), recent discoveries support the notion that cellular senescence plays a direct role in mammalian aging. In mouse models, p16 deficiency partially prevented the age-induced decline in cell proliferation and tissue function (Kim and Sharpless, 2006).

I.C.- Overexpression of oncogenes

Oncogenes such as Ras and Raf are usually regarded as agents of neoplastic transformation (Zhu et al., 1998). However, overexpression of the oncogene, Ras, or its downstream effectors can induce cellular senescence (Cristofalo et al., 2004). This effect may involve a tumor suppressive mechanism, i.e. the cells must prevent uncontrolled proliferation due to the aberrant activation of proliferation-driving oncogenes (Ben-Porath and Weinberg, 2005). The overexpression of oncogenic Ras has been shown to lead to permanent G1 arrest in primary human or rodent cells (Serrano et al., 1997). The fact that this cell cycle arrest by Ras is accompanied by accumulation of p53 and p16 suggests that Ras-induced senescence is dependent on the expression of p53 and p16. In fact, the inactivation of either p53 or p16 prevents Ras-induced cell cycle arrest in rodent cells (Serrano et al., 1997). In addition to the induction of senescence by overexpression of Ras, the overexpression of its downstream effector, Raf, also induces senescence (Zhu et al., 1998). It is shown that the activation of Raf-1 in non-immortalized human lung fibroblasts leads to a prompt and irreversible arrest of cellular proliferation and the premature onset of senescence. The induction of the CDK inhibitors p21 and p16 is observed in this proliferation arrest. However, the expression of p53 and p21 is not required for the Raf-induced senescence, as shown by the observation that ablation of p53

and p21 does not prevent senescence. This observation indicates that p16 plays a key role in Raf-induced senescence.

It is believed that extremely high levels of Ras and its downstream effectors are required for the induction of senescence. However, the expression of some mutant isoforms of Ras could trigger the opposite process, which is cell transformation. It has been observed that the conditional expression of an endogenous K-Ras (G12D) allele in the mouse tumor model does not induce senescence but rather enhanced proliferation and partial transformation (Tuveson et al., 2004). In contrast, in the Spitz nevi (a benign lesion which overlaps histopathologically with a melanoma), mutated multiple copies of HRAS oncogenes result in senescence (Maldonado et al., 2004). It is believed that elevated activation of the MAP-kinase pathway is responsible for HRAS-induced senescence in these lesions. The expression of p16 is greater in these cells than in those with only a single copy of mutated oncogene HRAS. Therefore, p16 functions as an essential mediator of oncogene-induced senescence which prevents the cells from progressing to melanoma.

Oncogene-induced cellular senescence also involves sequential signaling pathways. It has been shown that following the activation of MEK and ERK, the expression of the oncogene Ras results in the accumulation of active MKK3/6 and p38 which subsequently induces senescence (Wang et al., 2002).

I.D.- DNA damage

Previous studies show that cellular senescence can also be induced by DNA damage either through specific drugs or irradiation (Poele et al., 2002; Wahl and Carr, 2001). It

has been suggested that DNA damage could be a common causative agent underlying different forms of cellular senescence, including telomere shortening and oncogene-induced senescence (d'Adda di Fagagna et al., 2003). Thus, a certain degree of overlap does exist between the different mechanisms that have been implicated in the senescence phenotype.

The extent of DNA damage which occurs within cells increases with age. It has been observed that ageing correlates with significant increase in DNA mutations, DNA oxidation, and chromosome losses (Collado et al., 2007). It is assumed that age-related accumulation of DNA damage could lead to the accumulation of senescent cells in different tissues.

The ability of cells to proliferate requires an active DNA repair system. Inactivation of DNA repair genes such as XRCC4 in the mouse germline induces the premature senescence of cultured MEFs (te Poele et al., 2002). This premature senescence can be prevented by p53 inactivation, indicating that p53 plays a crucial role in DNA damage-induced senescence. p53 responds to DNA damage by activating transcription-dependent and transcription-independent pathways that result in cell cycle arrest or apoptosis, preventing proliferation of cells with damaged genome (te Poele et al., 2002). However, whether the cells undergo apoptosis or cell cycle arrest after p53 induction depends on a variety of factors, such as cell type, Rbs status, the expression of oncogenes and tumor suppressors. $p14^{ARF}$ has been shown to be involved in the stabilization of p53 following DNA damage and also plays a role in the long term maintenance of p53 activity after the induction of damage (Khan et al., 2004). DNA damage can also activate Rb via the p16 pathway to induce senescence (Shapiro et al., 1998).

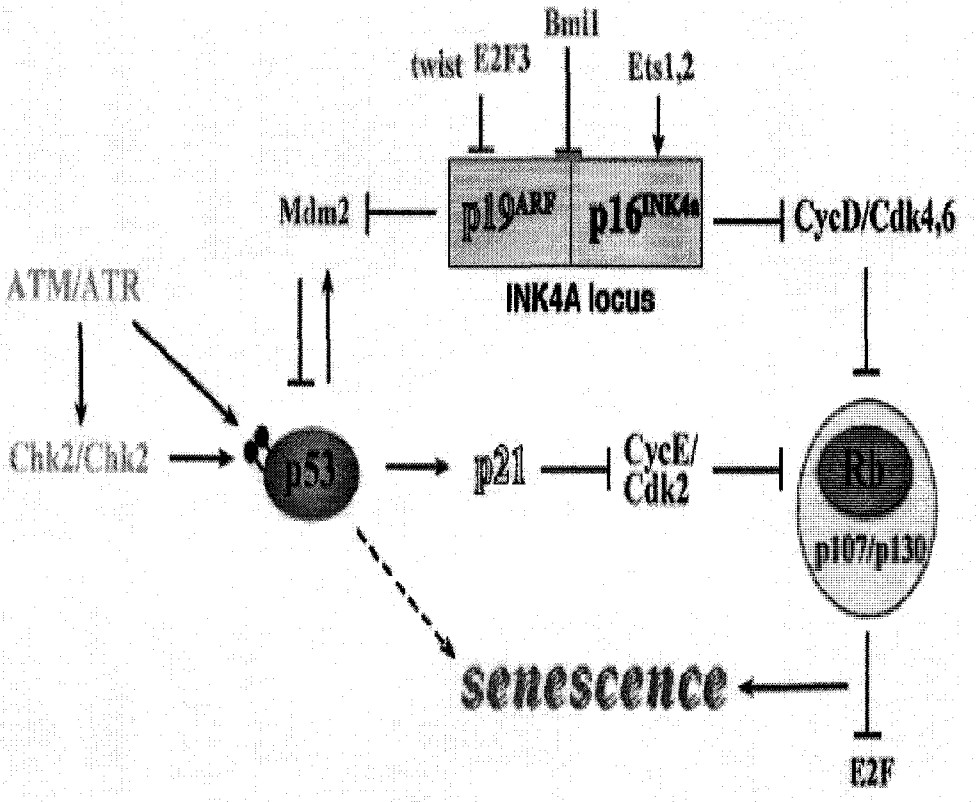
I.E.- Chromatin structure remodeling

One common feature of senescence is widespread nuclear heterochromatinization in which chromatin condenses causing chromosome to form punctuate heterochromatic foci called a senescence-associated heterochromatin focus (SAHF) (Adams, 2007). The histones present in these foci are posttranslationally modified and associate with protein ligands such as methylated lysine 9 of histone H3 (H3K9Me), heterochromatin protein 1 (HP1), histone variant macroH2 and other proteins less typically linked to heterochromatin such as high mobility group A (HMGA). Proliferation-promoting genes such as cyclin A are also present in heterochromatin and their silencing results in the induction of senescence (Adams, 2007). Since chromatin remodeling is associated with senescence, it is likely that chromatin structure remodeling can have a causative effect in cellular senescence. It has been shown that histone deacetylase (HDAC) might act as a mediator of early chromatin remodeling events and its overexpression leads to cellular senescence (Bandyopadhyay et al., 2007). The proliferation and differentiation of stem cells are controlled by key transcriptional factors whose activity is regulated by chromatin remodeling factors (Napolitano et al., 2007). The forced expression of brahma related gene 1 (Brg1), an essential component of chromatin remodeling complexes, induces significant cell cycle arrest in mesenchymal stem cells, associated with activation of the the p53 and pRb pathways. LKB1, a serine-threonine kinase, is found to associate with Brg1 and be necessary for Brg1-induced senescence (Marignani et al., 2001). However, cyclin E is found to be able to block Brg1 induced senescence (Shanahan et al., 1999). The involvement of p53 and pRb in chromatin remodeling induced senescence was confirmed by Kang et al. (Kang et al., 2004) who demonstrated that the interaction of

Brg1 with pRb is required for regulation of cell cycle progression in a p21 dependent manner. The involvement of p16 in chromatin remodeling-induced senescence has also been observed (Oruetxebarria et al., 2004). Oruetxebarria et al. have shown that malignant rhabdoid tumors (MRTs) lack the chromatin-remodeling factor hSNF5. Restoration of hSNF5 expression in these tumours induces the transcriptional activation of p16 leading to cellular senescence.

II- Tumor suppressing Pathways in senescence

Although many stimuli can induce cellular senescence, there are two main pathways for establishing and maintaining the senescent phenotype: p53 and pRB (Krtolica and Campisi, 2002) (Figure A). The importance of p53 in cellular senescence is emphasized by the observation that the inactivation of p53 in some replicatively senescent human cells can completely reverse senescence independently of the length of the telomeres (Beauséjour et al., 2003). The upstream regulator of p53 is p14 (p19 in mouse). p14 regulates p53 stability by inactivating the p53-degrading ubiquitin ligase MDM2 (Gil and Peters, 2006). p21 is a downstream target of p53 transactivation and a cyclin-dependent kinase (Cdk) inhibitor. Inactivation of p21 results in the ability of cells to bypass telomere-dependent replicative senescence (Brown et al., 1997). However, the mouse embryo fibroblasts lacking p21 are not immortal (Pantoja and Serrano, 1999). Therefore, other downstream targets of p53 are also responsible for p53-dependent induction of replicative senescence. A recent study has shown that the plasminogen activator inhibitor-1 (PAI-1) is a critical downstream target of p53 in the induction of replicative senescence (Kortlever et al, 2006). It has been shown that knockdown of PAI-1 results in the escape



(Ben-Porath and Weinbergk, 2005)

Figure A The molecular circuitry of senescence. p53 and Rb are the main activators of senescence. p53 can activate senescence by activating Rb through p21 and other unknown proteins, and also, in human cells, can activate senescence independently of Rb. Rb activates senescence by shutting down the transcription of E2f target genes. Rb is activated either by p21, or by the p16INK4a product. p53 activation is achieved by phosphorylation, performed by the ATM/ATR and Chk1/Chk2 proteins, and by the p19ARF product of the INK4a locus, which sequesters Mdm2 in the nucleolus. The transcriptional control of the INK4a products is not fully elucidated, indicated are some of these regulators.

from replicative senescence while its ectopic expression in proliferating p53-deficient fibroblasts activates the senescence phenotype. Another tumor suppressor, pRB, is regulated by p16 (Ohtani et al., 2004). p16 inhibits the formation of the complex of cyclin D-Cdk4/6 which is responsible for the phosphorylation and inactivation of pRB. The phosphorylation of pRB is required for the release of E2F transcriptional factors which promote the expression of late G1 genes whose products are required for S-phase initiation and progression.

The CDK inhibitors p21 and p16 are the two most studied markers of senescence. Although p21 is thought to be involved in the initiation of senescence, p16 is necessary for maintaining the senescent phenotype (Stein et al., 1999; Ben-Porath and Weinberg, 2005). Two known transcriptional factors involved in the regulation of p16 expression are Ets1 and Bmi1 (Collado et al., 2007). The expression of Ets1 which is a positive regulator of p16, increases significantly during mouse ageing while Bmi1, a potent repressor of p16, decreases in old cells. The p16-induced senescence is mediated by the p38MAPK pathway. ROS activates the expression of the INK4a/ARF locus via the p38MAPK. Therefore, the upregulation of p16 in senescence is likely due to the increase of oxidative stress in ageing cells. To date, two types of senescence *in vitro* have been observed: reversible senescence mediated by the activation of p53 and irreversible senescence.

III- Effect of senescence on key cellular processes

III.A.- Signal Transduction pathways

Previous studies have shown that several signaling pathways are impaired in senescent cells. In order to respond to mitogenic signals, cells require the activation of two major signaling cascades, the PI-3 kinase/PKB(Akt) and Raf/MEK/ERK pathways (Cristofalo et al., 2004). These pathways are activated in response to the association of SH-domain containing proteins with activated growth factor receptors. It has been shown that both the PI-3 kinase and the ERK pathways (required for cell proliferation) are impaired in senescent cells (Cristofalo et al., 2004). PI-3 kinase is a lipid kinase activated in response to phosphorylation of its p85 regulatory subunit by tyrosine kinase receptors (TKR). The functions of PI-3 kinase are due to its ability to catalyze the formation of the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3) which activates the atypical isoforms of the PKC family. PIP3 is required for the activation of PDK-1 which phosphorylates PKB/Akt and $p70^{s6k}$ (Toker and Cantley, 1997; Stephens et al., 1998; Pullen et al., 1998). Active $p70^{s6k}$ phosphorylates the ribosomal S6 protein and thus regulates the translation of mRNAs containing polypyrimidine tracks, including various components of the protein translation machinery (Brown and Schreiber, 1996). It has been shown that site-specific phosphorylation of $p70^{s6k}$ at Thr421/Ser424 is diminished in senescent cells, accompanied by a pronounced decrease in the phosphorylation of the ribosomal S6 protein (Zhang et al., 2000). In addition, the reduced abundance of active ERK molecules in cell nuclei is also observed in senescent cells (Kim et al., 2000). The declined nuclear abundance of phosphorylated ERK in senescent cells is thought to lead to the failure of ERK to activate nuclear targets required for cell cycle progression. Interestingly, it is assumed that proteolysis may regulate ERK activity directly by altering its protein stability and indirectly by controlling the stability of ERK phosphatases (Brondello et al., 1999).

III.B.- Apoptosis in cellular senescence

Apoptosis is a highly conserved process of programmed cell death whereby dying cells are removed by scavenging cells (Ellis et al., 1991). The mitochondria are the main regulatory centers of apoptosis due to its influence on the p53 and the Bcl-2 family of proteins, such as Bax and Bcl-2 (Siu and Alway, 2005). It is hypothesized that the ratio between the pro-apoptotic Bax and the anti-apoptotic Bcl-2 proteins determines whether the mitochondrial releases apoptotic factors such as cytochrome c and AIF into the cytosol thus inducing death (Green and Reed, 1998). Previous studies have shown that apoptosis is involved in the ageing process of various tissues such as those of the nervous and immune systems (Ohshima, 2006). It has been shown that the activation of the apoptotic pathway increases with age in rat skeletal muscle (Chung and Ng, 2006). This study demonstrated that in senescent rat muscle, the expression of Bax, Bcl-2 and Apaf-1 increases significantly during ageing. Furthermore, the levels of pro-caspase-12 and -17 as well as cleaved caspase-9 also increase in these senescent rat muscle cells. It has been also suggested that apoptotic and senescent cells present common features such as caspase activation and the binding of Annexin V to phosphatidylserines (Ohshima, 2004).

The main impact of apoptosis is to kill and eliminate potential cancer cells. However, this death pathway may also lead to the exhaustion of the number of division-competent stem cells. Such a quantitative loss of stem cells might be an important contributor to ageing (van Heemst et al., 2007). Over time, when cell loss starts to exceed cell renewal, impaired physiological functioning and ageing might result. An illustrative example of this is the greying of hair, which was found to follow loss of melanocyte stem cells and melanocytes in mice (Nishimura et al., 2005). Many studies detected an upregulation of

the expression of pro-apoptotic genes in ageing tissues, and a down regulation of the expression of anti-apoptotic genes (van Heemst et al., 2007). Likewise, in several human tissues, an increase in cell death by apoptosis was observed during ageing or age-related diseases, which was linked to increased expression of pro-apoptotic genes and/or decreased expression of anti-apoptotic genes (Joaquin and Gollapudi, 2001). The immune system represents one of the human organ networks that illustrates this aspect the most (Ginaldi et al., 2000). During human ageing, lymphocytes become increasingly sensitive to apoptosis and increase expression of pro-apoptotic proteins including death receptors and Bax, while decreasing expression of Bcl-2 (Gupta, 2000). It is tempting to speculate that a decreased proliferative potential of the immune system, caused by apoptosis, is responsible for the declined immune function observed in the elderly. Apoptosis has also been linked to other age-related pathologies, such as Alzheimer's disease, myocardiopathy, and sarcopenia (van Heemst et al., 2007).

III.C.- Translation

Three major and tightly regulated events are involved in protein synthesis: initiation of mRNA translation, elongation of the polypeptide chain and termination of mRNA translation (Gingras et al., 1999). Initiation is the rate-limiting step in mRNA translation and is thus the most common target of translational control. Signaling pathways such as those involving the insulin/insulin growth factor 1 (IGF-1), the kinase target of rapamycin (mTOR) and the p38 mitogen-activated protein kinase (MAPK) are believed to play crucial roles in the control of protein synthesis by targeting several components of the translation machinery (Wang et al., 1998). Global control of protein synthesis is generally realized by changes in the phosphorylation state of initiation factors or their regulators (Gebauer and Hentz, 2004).

There are contradictory reports in literature in regards to protein synthesis in cellular senescence or ageing. It is generally thought that the rate of protein synthesis decreases during ageing and senescence (Makrides, 1983). The decreased rate of protein synthesis has been reported for late passage senescent human embryonic fibroblasts (Ballard and Read, 1985), human foreskin fibroblasts from donors of different ages (Chen et al., 1980) and chick embryo fibroblasts (Macieira-Coelho and Lima, 1973). These results seem to be consistent with the observation that the activity of the eukaryotic initiation factor 2 (eIF2) and the eukaryotic elongation factor 1 (eEF-1) declines with age (Tavernarakis, 2007; Hussain and Ramaiah, 2007). However, it is not known whether the observed changes in protein synthesis are simply a consequence of ageing or if it plays a causative role in the induction of senescence (Tavernarakis, 2007). Several others studies have reported that the rate of protein synthesis is not altered during cellular senescence and ageing (Shakespear and Buchanan, 1976) (Hata, 1990). Therefore, the effect of senescence on general translation is presently controversial and thus unresolved.

IV-Cellular senescence and organismal ageing

The importance of senescence in the ageing process is evidenced by the fact that senescent cells accumulate in ageing tissues thus compromising their functionality (Krtolica and Campisi, 2002; Collado et al., 2007). Moreover, senescence may contribute to ageing by limiting the regenerative potential of stem cell pools (Collado et al., 2007). Therefore, by studying senescent cells, we may finally find a way to interfere with the ageing process.

V-Cellular senescence and late-life cancer as well as age-related diseases

Cellular senescence is a powerful tumor suppressive mechanism (Krtolica and Campisi,

2002). The oncogenic transformation of human cells *in vitro* is dependent on the loss of the activity of several tumor suppressor factors (Hahn and Weinberg, 2002). In fact, tumor suppressor activities are significantly reduced in human cancer cells (Gil and Peters, 2006). However, senescent cells may contribute to the development of cancer by altering the microenvironment of cells and tissues in aged individuals. Under these circumstances, the secretion of extracellular factors stimulates the pre-malignant and malignant proliferation of epithelial cells resulting in the formation of tumors (Krtolica et al., 2001). In addition, senescent human lung fibroblast cells have been shown to promote tumorigenesis in mice (Coppe et al., 2006; Parrinello et al., 2005). Therefore, cellular senescence is thought to be an example of antagonistic pleiotropy: protecting organisms from cancer early in life, but promoting cancer later in life (Krtolica and Campisi, 2002). Senescent cells may also contribute to age-related diseases due to the observation that senescent cells accumulate at sites of age-related pathology. For example, compared with cells from unaffected areas, cells cultured from venous ulcers have a reduced replicative life span and increased proportion of β-galactosidase positive cells (Krtolica and Campisi, 2002). Therefore, understanding the nature of senescent cells is important since it may enable the identification of novel approaches needed to combat late-life related cancers and other age related diseases.

VI- Effect of senescence on cell response to stresses

Senescent cells have a decreased capacity to adapt to environmental stresses (Helenius et al., 1999; Bruunsgaard and Pedersen, 2000). Many studies have been done on the response of senescent cells to different stresses (oxidative stress, heat stress, UV stress,

Table A Stress responses of senescent cells or organisms

stress	Response	Reference
Irradiation	Absence or impairment in recovery response in senescent cells	Romani et al., 1968 Helenius et al., 1999
Oxidative	Senescent cells are resistant to apoptosis induced by H2O2	Ryu et al., 2007
Heat	Hsp70 protein and gene are decreased in senescent cells	Effros et al., 1994 Luce et al., 1992 Hall et al, 2000 Wu et al., 1993
UV	Senescent cells show impairment in signaling pathways	Helenius et al., 1999 Adler et al., 1996
Mechanical	Senescent cells and organism show an increase in pro-inflammatory proteins and decrease in protooncoproteins	Shimizu et al., 2000 Miura et al., 2000 Klein-Nulend et al., 2002 Always, 1997
Chemical	Senescent cells are resistant to apoptosis induced by staurosporine, thapsigargin and MG132	Ryu et al., 2006 Kraft et al., 2006
Nutritional	Senescent mice liver shows attenuated increase in oxidative stress and pro-inflammatory protein; senescent cells also show declined ability to mount the proteolytic response;	Seo et al., 2006 Taylor et al., 1991
Hemodynamic	Senescent rat heart shows decreased induction of protooncogenes and heat shock genes;	Isoyama, 1996 Shida and Isoyama, 1993
Immobilization	Senescent rats show declined induction of Hsp70	Blake et al., 1991 Udelsman et al., 1993
Environmental	After chronic overcrowding, senescent rat shows decreased immune response but another study shows that senescent rat has no significant change in immune response under stress;	Csermely et al., 1995 Odio et al., 1989
Exercise	Acute high intensity exercise results in mobilization of senescent T lymphocytes and chronic exercise leads to decreased expression of Hsp70 in senescent rat;	Simpson, 2007 Naito et al., 2001
Combined stresses	caloric restriction and lifelong exercise leads to attenuated increase in oxidative stress and pro-inflammatory protein in senescent mice; restraint stress and vitamin E do not prevent immunological decline in senescent mice;	Seo et al., 2006 Wakikawa et al., 1999

mechanical stress, chemical stress, nutritional stress, hemodynamic stress, immobilization stress, exercise stress, environmental stress and combinations of two or three stresses) (Table A). The stress response of senescent cells and aged organisms is different from their young counterparts in many aspects outlined below:

VI.A.- Effect of senescence on cell response to apoptotic inducers

The reports on the response of senescent cells to stress-induced apoptosis are contradictory. It has been shown that senescent cells are resistant to apoptosis induced by H_2O_2 and staurosporine due to elevated levels of the high focal adhesion kinase (FAK) and its Src-dependent phosphorylation on the Try577 residue (Ryu et al., 2007). Yeo *et al.* have furthermore demonstrated that senescent human fibroblasts are resistant to UV-induced cell death (Yeo et al., 2000) due to inhibition of the SAPK/JNK pathway. The decreased SAPK/JNK activity was closely linked to increased levels of the p21 protein, which is thought to protect cells from apoptotic cell death. In addition, senescent human skin fibroblast cells exhibit less cell death in response to high doses of the ubiquitin-proteasome inhibitor MG132 (30μM) than young cells (Kraft et al., 2006). Senescent human diploid fibroblast cells are also resistant to thapsigargin or serum withdrawal induced apoptosis due to elevated Bcl-2 levels in these cells (Ryu et al., 2006; Wang et al., 1995). Additionally, senescent fibroblasts resist stress-induced apoptosis as a result of decreased expression and/or activation of caspase-3 and p53 (Marcotte et al., 2004; Seluanov et al., 2001).

Several other groups, however, have reported that senescent cells are not resistant to stress-induced apoptosis. It has been shown that senescent human fibroblasts are susceptibility to ceramide, TNFα or okadaic acid induced apoptosis. (DeJesus et al., 2002). In addition, it has been shown that T cell prepared from ageing donors are prone to

anti-Fas-induced apoptosis (Aggarwal and Gupta, 1998). In this study, the authors observed an increased expression of Fas and Fas ligand and a decreased expression of Bcl-2 in both CD4+ and CD8+ T cells from ageing human compared with young controls. Bax expression also increased in ageing lymphocytes at both the mRNA and protein levels. Subsequently, they found that during anti-Fas treatment, there is an increase in the cleavage of caspase-8 and caspase-3 in the aged lymphocytes. These and other observations, prompted the authors to suggest that the alteration in the expression and activity of molecules in Fas/FasL signaling pathway can play a role in the increased Fas-mediated apoptosis in ageing human (Aggarwal and Gupta, 1999).

VI.B.- Effect of senescence on cell recovery from stress

After treatment of stress, senescent cells recover more slowly than young cells. It has been reported that limb immobilization causes rapid and significant muscle weight loss. The loss can be overcome efficiently in the muscles of young animals. However, muscles of old animals do not recover as well, suggesting slower muscle turnover (degradation and synthesis of proteins) (Zarzhevsky et al., 2001). Rosenfeldt *et al.* showed that ageing hearts have a diminished capacity to recover from stress (Rosenfeldt et al., 2004). Upon exposure to oxygen, early passage diploid cells repair the oxygen-induced damage in contrast to late passage cells which fail to do so (Honda and Matsuo, 1987).

VI.C.- Effect of senescence on signal transduction pathways

The differential activation of signaling pathways in stressed proliferative versus senescent cells is also ambiguous. Some reports show that cell signaling pathways are impaired in senescent cells under stresses while other reports show the opposite results.

After UV treatment, senescent human fibroblasts show a great attenuation in the induction of the NF-kappaB-mediated signaling pathway (which is a major defense system against environmental stresses) (Helenius et al., 1999). This attenuation affects mostly the DNA binding activity of nuclear NF-kB complexes. In addition, old human dipoid fibroblasts cells treated with heat shock and UVC light show decreased activation of the JNK pathway compared to young cells (Adler et al., 1996). Senescent human fibroblasts have been shown to have decreased SAPK/JNK activity after UV treatment (Yeo et al., 2000). However, under repeated mechanical tension force, senescent human periodontal ligament derived fibroblast (hPLF) cells show an increase in Plasminogen Activator (PA) activity. PA converts plasminogen to plasmin which activates the kinin cascade and latent extracellular matrix metalloproteases (Miura et al., 2000).

VI.D.- Senescence and cell response to oxidative stress

Upon exposure to various stresses, senescent cells produce free oxygen radicals which mediate an oxidative response. Senescent human fibroblast cells treated with H_2O_2 stress are vulnerable to the accumulation of oxidized proteins and do not remove these proteins as efficiently as young cells (Merker et al., 2000). Under UVA stress, senescent human skin fibroblasts show a decrease in the expression of some antioxidant system (Hazane et al., 2005). Starke-Reed and Olivier have furthermore shown that under oxidative stress (when treated with 100% oxygen), the level of oxidized proteins continues to increase in senescent rats due to the fact that the activity of alkaline protease which degrades oxidized proteins does not increase in comparison to young rats (Starke-Reed and Olivier, 1989). However, the combination of some stresses can decrease the accumulation of oxidized proteins in senescent cells. Restricting caloric intake combined with lifelong

exercise can attenuate the oxidative response thus protecting the senescent cells. (Seo et al., 2006). The protective function of caloric restriction in aging is due to decreased methyglyoxal (MG) which is formed by glycolytic intermediates and glycates proteins. MG damages mitochondria and induces pro-oxidative state. The effect of caloric restriction is also seen by the observation that mitochondria thioredoxin reductase (TrxR2) in skeletal and heart muscle of old rat is back to normal levels after caloric restriction (Rohrbach et al., 2006). It is interesting that exercise alone can also improve the anti-oxidant system. It has been shown that oxidative stress can be decreased and anti-oxidant defense can be increased in old rats by exercise (Asghar et al., 2007).

VI.E.- Senescence and heat shock proteins

Heat shock proteins are involved in the protection of cells from injury and recovery from stress (Kregel et al., 1995). Synthesis of Hsp70 is an important component of the overall physiological response to stress (Hall et al., 2000). Hsp70 is known to protect cells from the detrimental effects of heat shock. This was evidenced by Riabowol *et al* who demonstrated that cells were unable to survive a brief incubation at 45°C when they were microinjected with antibodies against Hsp70 (Riabowol et al., 1988). Previous in vitro and in vivo studies have shown that the expression of Hsp70 decreases in senescent cells exposed to heat shock (Effros et al., 1994; Luce and Cristofalo, 1992). The levels of transcription factors required for the transcription of the heat shock proteins are also affected in stressed senescent cells. Lee *et al.* have shown that the activity of the heat shock transcription factor (hsf) can be affected in stressed senescent cells (Lee et al., 1996). They demonstrated that the responsiveness of hsf1 to activation by heat stress is decreased in senescent cells.

In contrast to acute heat stress, repeated mild heat-shock (RMHS) is beneficial to senescent cells. The basal level of some heat shock proteins including Hsp70 is increased greatly in senescent human fibroblast cells after RMHS treatment, accompanied by the improved functional and survival ability of the cells (Fonager et al., 2002). RMHS also reduces the accumulation of oxidized and glycoxidized proteins in senescent human skin fibroblast cells and increases the expression of Hsp70 (Verbeke et al., 2001).

VI.F.- Inflammatory response of senescent cells

Previous studies have shown that the expression of pro-inflammatory proteins increases in senescent cells under stress. Senescent cells, when subjected to mechanical stress, increase the expression of prostaglandin E2 (PGE2), interleukin (IL)-1 beta and COX-2 (cyclooxygenase-2) (Shimizu et al., 2000; Klein-Nulend et al., 2002; Ohzeki et al., 1999).

The immune response of old cells or organisms can also decrease depending on the nature of the stress. Vitamin E, for example, has been shown to rescue the immunological decline seen in young mice but not in senescent mice (Wakikawa et al., 1999). Moreover, after acute maximal exercise, older individuals show a less resilient leukocytosis than young people (Ceddia et al., 1999). Killer). After chronic overcrowding, T cells from aged mice show significantly lower levels of resting and lectin-stimulated intracellular calcium concentrations than young mice. This suggests that the inadequate adaptation in calcium metabolism of T lymphocytes may contribute to the decreased immune response of senescent cells exposed to a variety of stresses (Csermely et al., 1995).

VI.G.- Effect of anti-stress drugs on old organisms

Treatment of old animals with some anti-stress drugs has been shown to beneficially protect these animals from various assaults. For example, acetyl-L-carnitine has been demonstrated to protect old rats from the effects of oxidative stress by inducing the expression of heme oxygenase-1, hsp70 and SOD-2 (Superoxide dismutase 2) (Calabrese et al., 2006). Furthermore, although the heart of old rat has a lower tolerance to aerobic stress, pre-treatment with coenzyme Q10 and rapid pacing after isolation significantly improves their tolerance to oxygen deprivation (Rowland et al., 1998). Treatment of these rats with Levocarnitine (which plays an important role in the beta-oxidation of fatty acid) increases the skeletal mitochondrial antioxidant system while decreasing the incidence of free radical-induced lipid peroxidation (Kumaran et al., 2003).

VI.H.- Induction of proto-oncogenes and cancer incidence in old organisms

The expression of protooncogenes decreases when senescent cells are subjected to various stresses. Upon exposure to hemodynamic stress, senescent heart cells express decreased levels of proto-oncogenes such as c-myc and c-fos (Isoyama 1996). Moreover, upon being subjected to various rounds of stretches, skeletal muscles of old quails show decreased expression of c-myc oncoprotein (Alway, 1997). However, these effects do not prevent cell transformations and tumor growth in old organisms.

Previous studies have shown that senescent rats have a more favorable condition for tumor growth than young rats under stress (Sapolsky and Donnelly, 1985). When these animals are inoculated with fetal rat cells that were transformed by tumor-induced viruses they develop tumors more rapidly than their young counterpart. This effect is mainly due to hypersecretion of corticosterone.

VII- Can Stress Granules be formed in Senescent Cells

In addition to the above-mentioned cell stress responses, recent advances also show that cells respond to stresses by forming cytoplasmic foci called stress granules (Kedersha and Anderson, 2002). However, we do not know whether stress granules can be induced in senescent cells. Stress granules can be induced by different stresses such as UV, heat shock, arsenite and inhibition of the ubiquitin-proteasome system (Mazroui et al., 2007 ; Mazan-Mamczarz et al., 2006; Gallouzi et al., 2001; David et al., 2007). Stress granules are cytoplasmic foci which harbor several RNA binding proteins (Table B) and their associated mRNAs. The localization of these mRNAs to the granules maintains them in a translationally repressed state until the cells can recover from the stress. Some of the RNA binding proteins present in the granules have been previously shown to affect the translation (TIA-1/R ; T-cell internal antigen-1/TIA related protein and FMRP;Fragile X Mental Retardation Protein) or stability/turnover (HuR, TTP and G3BP;Ras-GAP SH3 binding protein) of messages (Gallouzi et al., 2000; Stoeckling et al., 2004; Tourrière et al., 2003). Additionally, these granules are also known to contain several other proteins such as the 40S ribosomal subunit and the translation initiation factors eIF4E, eIF4G and eIF2 (Kimball et al., 2003). To date, stress granules are known to be induced by both eIF2α dependent and independent pathways (Kimball et al. 2003; Mazroui et al., 2006; Dang et al., 2006). The stress induced activation of one or more kinases known to induce the phosphorylation of eIF2α results in the formation of stress granules (Kedersha and Anderson, 2002). Upon phosphorylation of eIF2α, the levels of the eIF2-GTP-tRNA(Met) ternary complex (which is required to load the initiator methionine onto the 48 S preinitiation complex) become scarce. This, subsequently, leads to the binding of TIA-

Table B Proteins found in stress granules

protein class	proteins in stress granules	reference
ribosome	40S	Kedershan et al., 2002
translation	eIF2	Kedershan et al., 2002
	eIF3	Kedershan et al., 2002
	eIF4E	Kedershan et al., 2002
	PABP	Kedershan et al., 1999
	PCK/CGH-1	Wilczyhska et al., 2005
	TIA-1/R	Kedershan et al., 1999
RNA stability	TTP	Stoecklin et al., 2004
	HUR/D	Gallouzi et al., 2000
RNA binding proteins	Staufen	Thomas et al, 2004
	G3BP	Torriere et al., 2003
	CUGBP-1	Fujimura et al., 2007
	SMN	Hua and Zhou, 2004
	FMRP	Mazroui et al., 2002
Exonuclease	XRN1	Kedershan et al., 2005

1/TIAR to the 48 S complex, promoting polysome disassembly and the concurrent routing of mRNA into stress granules (Figure B). In the reported eIF2α phosphorylation independent pathway, interfering with the activity of eIF4A (which is an RNA helicase required for the ribosome recruitment phase of translation initiation) induces stress granule formation. In this pathway, blocking eIF4A activity does not induce the phosphorylation of eIF2α (Mazroui et al., 2006).

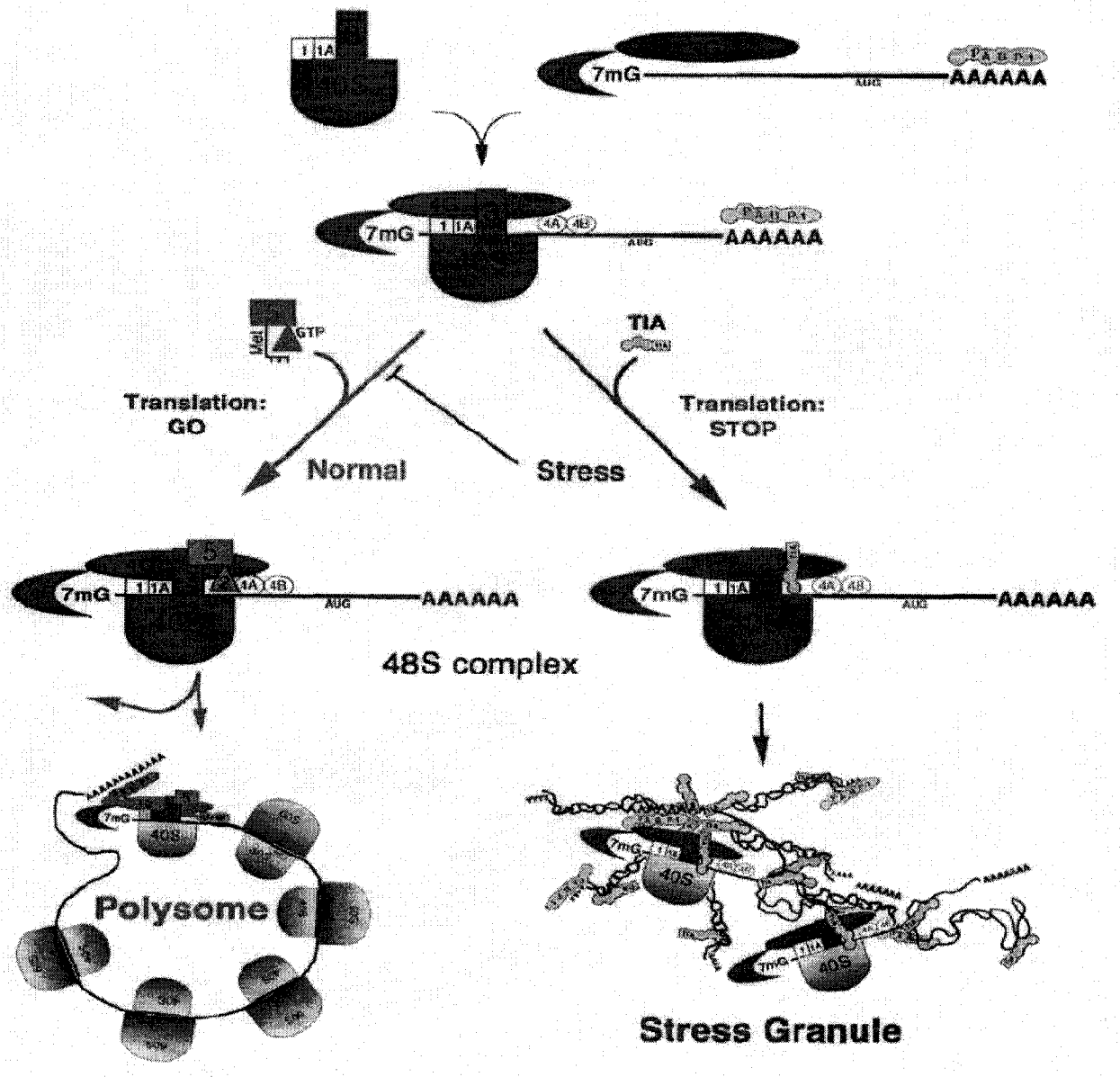
Although stress granules are formed under different stress conditions, they can also be induced by the overexpression of RNA binding proteins such G3BP and Caprin-1(Solomon, et al., 2007; Tourrière, et al., 2003). Stress granules can also be induced by the overexpression of other proteins such as the survival motor neuron protein (Hua and Zhou, 2004). Moreover, mouse hepatitis coronavirus have been shown to induce the formation of stress granules which results in the translational repression of host mRNAs. (Raaben et al., 2007).

The assembly of stress granules is a highly dynamic process in which translationally stalled mRNAs are sorted and processed for either degradation, storage or reinitiation (Kedersha et al., 2000). Stress granules can be disassembled by drugs which stabilize polysomes, such as emetine (Kedersha et al., 2000) and cycloheximide (Kedersha et al., 2005). They can also be disassembled in an Hsp70 dependent manner (Mazroui et. al, 2007). The interaction of TIA-1/TIAR with components of the West Nile virus has also been shown to inhibit the formation of stress granules (Emara and Brinton, 2007).

When cells are subjected to stresses, the general translation machinery is shut off and some messages are recruited to stress granules. The mRNAs recruited to stress granules are selective (Kedersha et al., 2002). Those mRNAs which are crucial for cell survival such as Hsp70 mRNA are not recruited to stress granules (Kedersha et al., 2002). Stress

Figure B Translational initiation in the absence or presence of stress

In the absence of stress, the eIF2–GTP–tRNAMet ternary complex (green) is available to form a canonical 48 S preinitiation complex at the 5« end of capped transcripts (green arrow: normal) and scanning begins. Upon recognition of the initiation codon by the anticodon of tRNAMet, eIF5 promotes GTP hydrolysis and early initiation factors are displaced by the 60 S ribosomal subunit. As additional ribosomes are added to the transcript, the mRNA is converted into a polysome (bottom left). In stressed cells, phosphorylation of eIF2*a* depletes the stores of eIF2–GTP–tRNAMet, and the cytoplasmic amount of TIA-1 (yellow) increases. Under these conditions, TIA-1 is included in a noncanonical, eIF2/eIF5-deficient preinitiation complex that is translationally silent. TIA-1 auto-aggregation then promotes the accumulation of these complexes at discrete cytoplasmic foci known as SGs (composed of all components of the 48 S pre-initiation complex except eIF2 and eIF5). Translational components that are present in SGs are shown in red; non-translation proteins present in SGs are yellow, those absent from SGs are shown in green.



(kedersha and Anderson, 2002)

granules also play a role in the post-transcriptional regulation of mRNAs. Some messages are stabilized by stress granules, such as p21 mRNA (Mazroui et al., 2007). However, we do not know how the post-transcriptional regulation by stress granules may have an influence on senescent cells.

The reported studies about stress granules have all been performed on proliferative cells. Since senescent cells are more prone to stresses than young cells, it will be interesting to investigate whether stress granules can be induced in senescent cells and how these granules may mediate the response of senescent cells to stresses.

Summary

Cellular senescence is the state in which cells arrest grwoth and not responsive to mitogen stimuli. Cellular senescence can be induced by different mechanisms and is maintained mainly by two tumor suppressing pathways involving the proteins p53 and pRb. The downstream transactivating target of p53 is p21 while the upstream regulator of p53 is p14. p14 stabilizes p53 by preventing its binding to MDM2 which mediates the degradation of p53. p16 positively regulates pRb by blocking the formation of the complex cyclin D2-Cdk4/Cdk6 which phosphorylates and inactivates pRb. Increasing evidence indicates that cellular senescence may contribute to aging as well as to late-life cancers. Senescent cells show a declined adaptive capacity to different environmental stresses. Although many studies show that proliferative cells respond to stresses by forming cytoplasmic foci called stress granules, no reports are available on the induction or the functional relevance of stress granules during senescence. Therefore, the purpose of

this study is to investigate whether stress granules can be induced in senescent cells and how they may mediate the response of senescent cells to stresses.

Rationale

Senescent cells secrete high levels of toxic factors (such as matrix metalloproteinases (MMPs), epithelial growth factors and inflammatory cytokines) into its microenvironment which can contribute to the development of cancer (Campisi, 2005; Krtolican and Campisi, 2002). These secreted substances can disrupt tissue integrity and functions, leading to cell death resistance and the progression of late-life cancers. It has been shown that senescent human fibroblast cells can stimulate pre-malignant and malignant proliferation of epithelial cells and tumor formation in mice by excreting extracellular factors (Krtolica et al., 2001). In addition, senescent human lung fibroblasts have been shown to promote tumorigenesis in mice (Coppe et al., 2006). Moreover, coinjection of premalignant mammary epithelial cells and senescent human fibroblasts into mice makes the premalignant cells more invasive and tumorgenic (Parrinello et al., 2005). Senescent cells may also contribute to age-related diseases due to the observation that these cells accumulate at sites of age-related pathology (Krtolica and Campisi, 2002). Therefore, it is important to delineate the molecular mechanisms by which senescent cells react and adapt to environmental changes. In fact, many studies have shown that senescent cells have a decreased adaptive capacity to environmental stresses (Helenius et al., 1999; Bruunsgaard and Pedersen, 2000). In response to certain stresses, cells have been shown to form cytoplasmic foci named stress granules. However, the formation and the functional relevance of these granules have been defined only in proliferative cells. Since senescent

cells in old organisms are also exposed to a variety of assaults, we asked whether stress granules can be induced in senescent cells and whether these entities may be involved in preventing the expression of factors which contribute to the development of late-life cancers. Our results described below show that stress granules can be induced in senescent cells. Furthermore, the number of stress granules increases during senescence. We also observed that under stress, p21 mRNA and the RNA-binding protein, CUGBP1, are recruited in stress granules, leading to a significant decrease in the expression of p21 protein.

Results

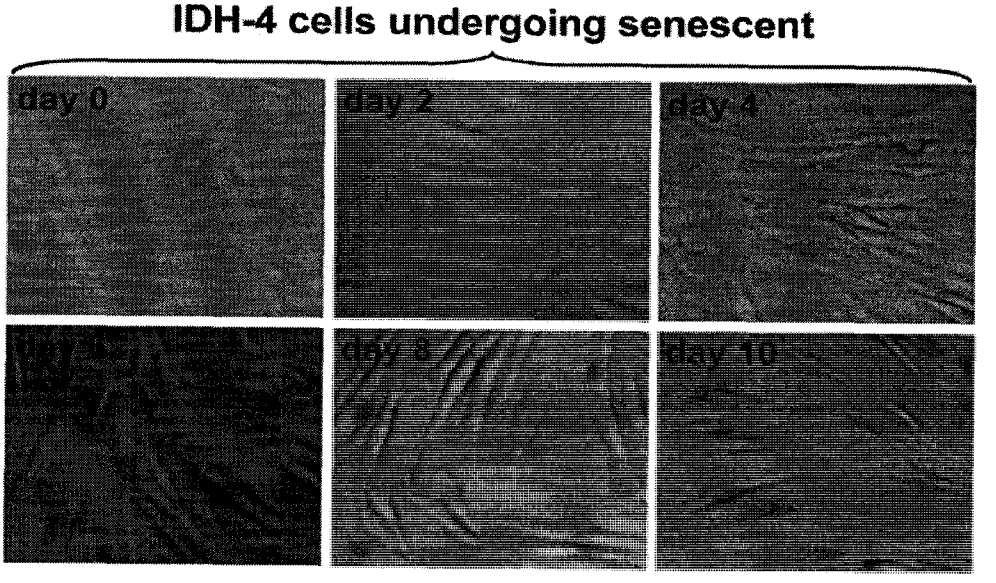
Senescent cells respond to stress by forming a high number of Stress Granules (SGs)

To investigate the formation and the role of stress granules (SGs) during senescence, we first followed their assembly in senescent cells exposed to a variety of stresses. We used two model systems: serial passage of WI-38 human diploid fibroblasts and IDH4 human fibroblasts, in which dexamethasone-induced expression of the SV40 large T antigen can overcome its normal limited life span (Wang et al., 2001). It is well-established that senescence can be induced in IDH4 cells by the removal of dexamethasone from their growth media which is replaced by charcoal stripped FBS and in WI-38 by a serial passages until they reach the population doubling (PD) number of 36 or more (Hayflick, 1985; Hayflick, 1998). One of the well-used biomarker for replicative senescence is senescence-associated- β -galactosidase (SA- β -gal) activity (Dimri et al., 1995). While testing senescence activation in our cell systems, we observed that >95% of IDH4 cells presented a SA-β-gal staining 8 days after the removal of dexamethasone (Figure 1A). The same β-gal staining was also obtained in WI38 fibroblasts as early as PD number 36 and was more pronounced at PD number 39 (Figure 1B). The serial passages and population doubling number for WI-38 cells have been determined as previously described (Wang et al., 2001).

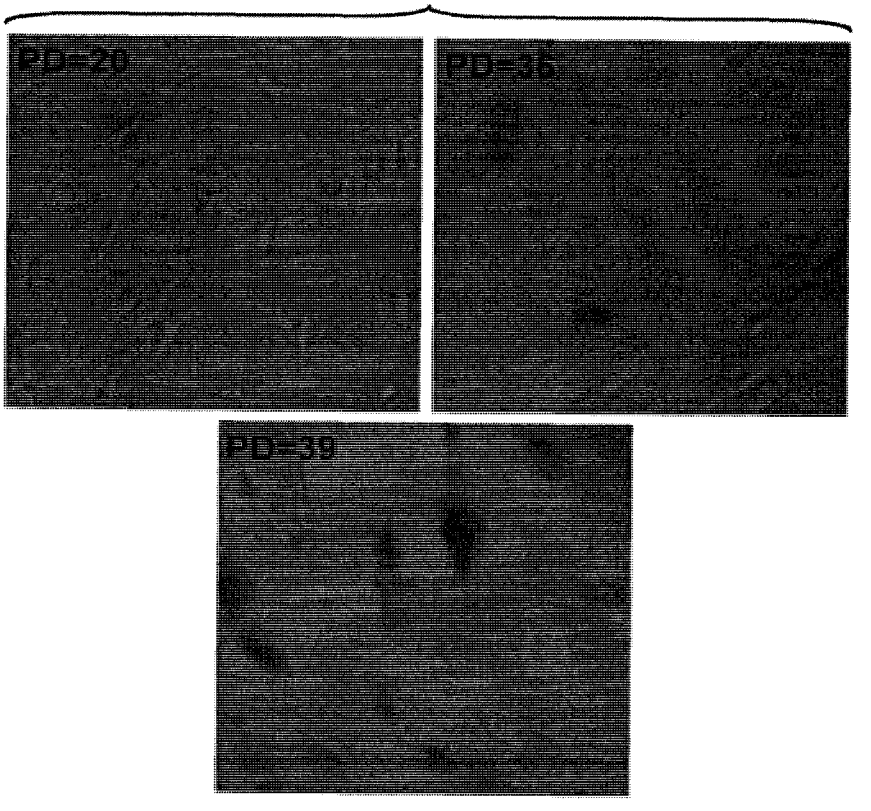
Several laboratories have shown that treatment of cells with oxidative stress-inducing drugs such as arsenite (AS) triggers the formation of SGs (Kedersha et al., 2005). It is also well established that proliferative and senescent cells differentially respond to oxidative stress (Merke et al., 2000; Servais et al., 2005; Starke-Reed and Olivier, 1989).

Figure 1. Models of senescence
A) IDH4 cells as a model of senescence. IDH4 cells grown in media with (day 0) and without dexamethasone (day 2, 4, 6, 8 and 10) are subjected to β-galatosidase staining at different time points. B) WI38 cells as a model of senescence. WI-38 cells were grown in normal media as described in the Materials and Methods section were subjected to serial passages. The cells with different population doubling numbers (PD) were subjected to β-galatosidase staining.

A

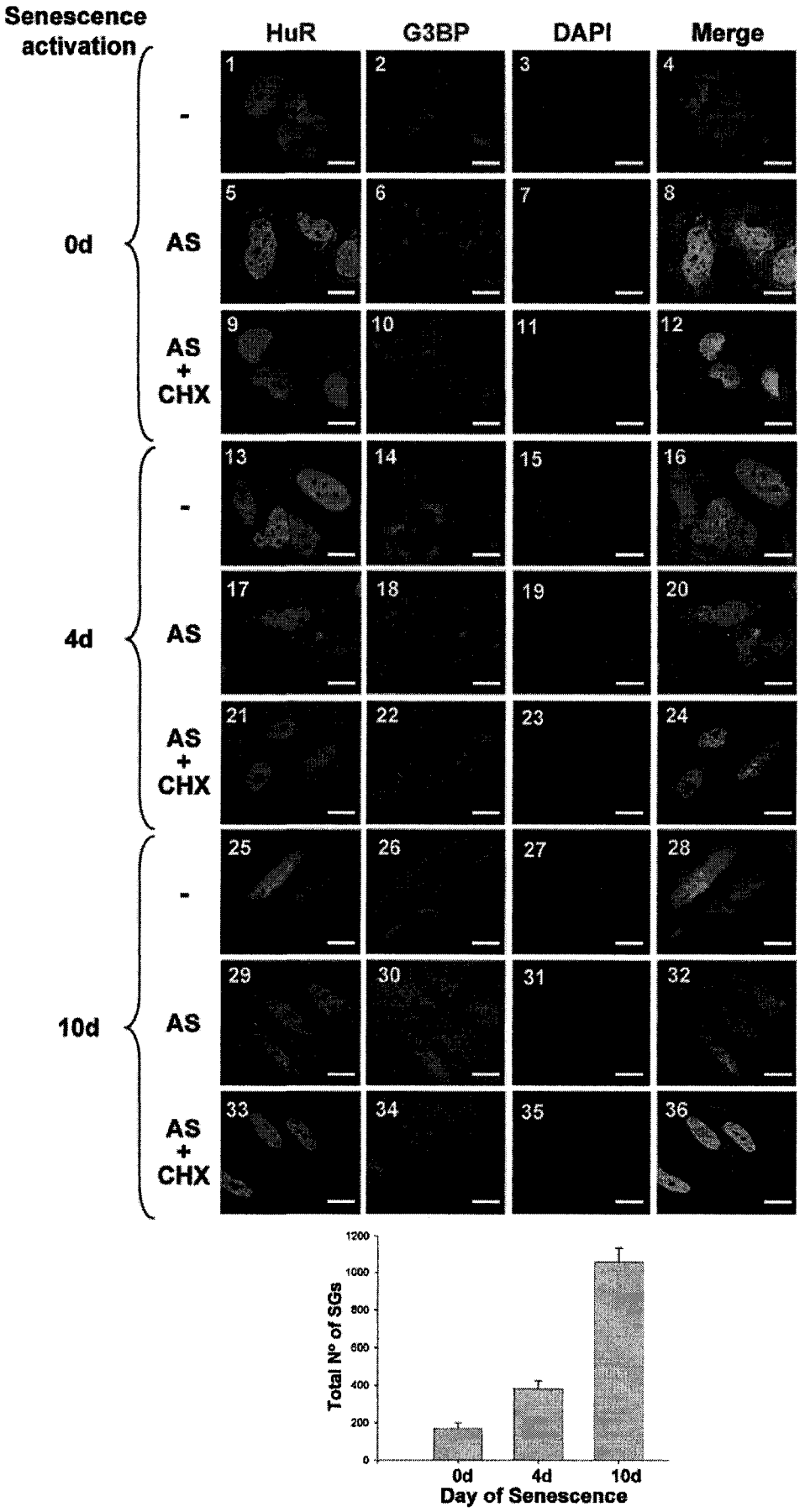

IDH-4 cells undergoing senescent
day 0
day 2
day 4
day 8
day 10

B


WI-38 cells undergoing senescent
PD=20
PD=35
PD=39

Thus, it was of high interest to determine whether SGs could assemble in senescent cells under these stress conditions. IDH4 cells grown in proliferative (day 0) or in senescent media (day 4 and 10 after dexamethasone removal and charcoal stripped FBS was used as the media) were exposed to 0.5 mM AS for 30 min (Figure 2). These cells were then fixed and immunofluorescence experiment was performed using antibodies against two well-established SGs markers, HuR (Gallouzi et al., 2000) and G3BP (Tourriere et al., 2003). As expected, AS -induced SGs were visible in both proliferative and senescent cells (Figure 2, compare panels 1, 2, 13, 14, 25, 26 to panels 5, 6, 17, 18, 29, 30). Their number was 5 fold higher in late senescent cells (day 10) when compared to their proliferative counterpart (Figure 2, graph, compare day 0 to day 10). In early senescent cells, however, the number of SGs increased by only 2 fold (Figure 2, graph, compare day 0 to day 4). Since, as expected (Wang et al, 2001), the levels of HuR protein significantly decreased during senescence (Figure 2), we defined the number of SGs using G3BP as a marker. In order to characterize these foci as bona fide SGs, we assessed their assembly in the presence or absence of cycloheximide (CHX) (Figure 2). CHX is an inhibitor of translation elongation which has been previously shown to trap mRNAs on polysomes causing the disassembly of SGs (Kedersha et al, 2005). We observed that the addition of CHX to AS-treated proliferative and senescent IDH4 cells prevented SGs formation (Figure 2, panels 9, 10, 21, 22, 33, 34). In order to verify if the increased number of SGs is a general attribute that can be detected in other senescent cell systems, we assessed their assembly in the WI-38 fibroblasts (Figure 3). As in IDH4 cells, the number of AS-induced SGs increased by ~4 fold in WI-38 cells at PD 39 (senescent) when compared to those at PD 20 (proliferative) (Figure 3). Additionally, we observed that SGs can also form in senescence cells when exposed to other stresses such as heat shock (Figure 4) or

Figure 2. Arsenite differentially induces the formation of stress granules in proliferative and senescent IDH4 cells. IDH4 cells at various time points (0d. 4d, 10d) after the induction of senescence were treated for 30 minutes with or without 0.5mM arsenite in the presence or absence of cycloheximide. Cells were subsequently fixed, permeabilized and were analyzed by immunofluorescence with antibodies against two surrogate markers of SGs: HuR and G3BP. The graph illustrates the number of stress granules formed (as evidenced by G3BP staining) on the different days of senescence. The number of stress granules in three random fields with 10 random cells was counted and the average calculated.


Senescence activation
HuR
G3BP
DAPI
Merge
0d
4d
10d
-
AS
AS + CHX
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
Total N° of SGs
0
200
400
600
800
1000
1200
0d
4d
10d
Day of Senescence

the proteasome inhibitor, MG132 (Mazroui et al., 2007) (data not shown). Together our data suggested that forming a high number of SGs is one of the early responses used by senescent cells to cope with a variety of stresses.

Senescent cells recover more slowly after removal of stress

Although, the data described above show that SGs can form in senescent cells, they do not give any indication on their behavior when the stress is removed. Previously, it has been shown that SGs disassemble 2 to 3h after the removal of stress (Mazroui et al., 2002). To test whether this could also be the case in senescent cells we assessed the presence of SGs at different time points (0h, 1h, 2h, 4h, 6h and 24h) after the removal of AS from the media of IDH4 cells grown in the presence (day 0) or absence (day 4 and 10) of dexamethasone. Immunofluorescence experiments using anti-G3BP and -HuR antibodies showed that the disassembly of SGs was complete ~2h hours after the removal of AS from proliferative and senescent IDH4 cells (Figure 5). Additionally, we observed that the increase in SGs number in late senescent cells (Figures 2-4) correlated with a small but consistent delay (from 2h to 4h) in their disassembly during cell recovery from stress (Figure 5, panels 33 to 48).

Recently, we reported that the disassembly of MG132-induced SGs occurs in an Hsp70-dependent manner (Mazroui et al., 2007). Since Hsp70 is also one of the key players during cell recovery from a variety of stresses (Kregel et al., 1995; Gabai and Sherman, 2002), we hypothesized that the observed increase in the number of AS-induced SGs in senescent cells could have an impact on its expression. To test this possibility, we investigated the expression of Hsp70 protein during the recovery of proliferative and

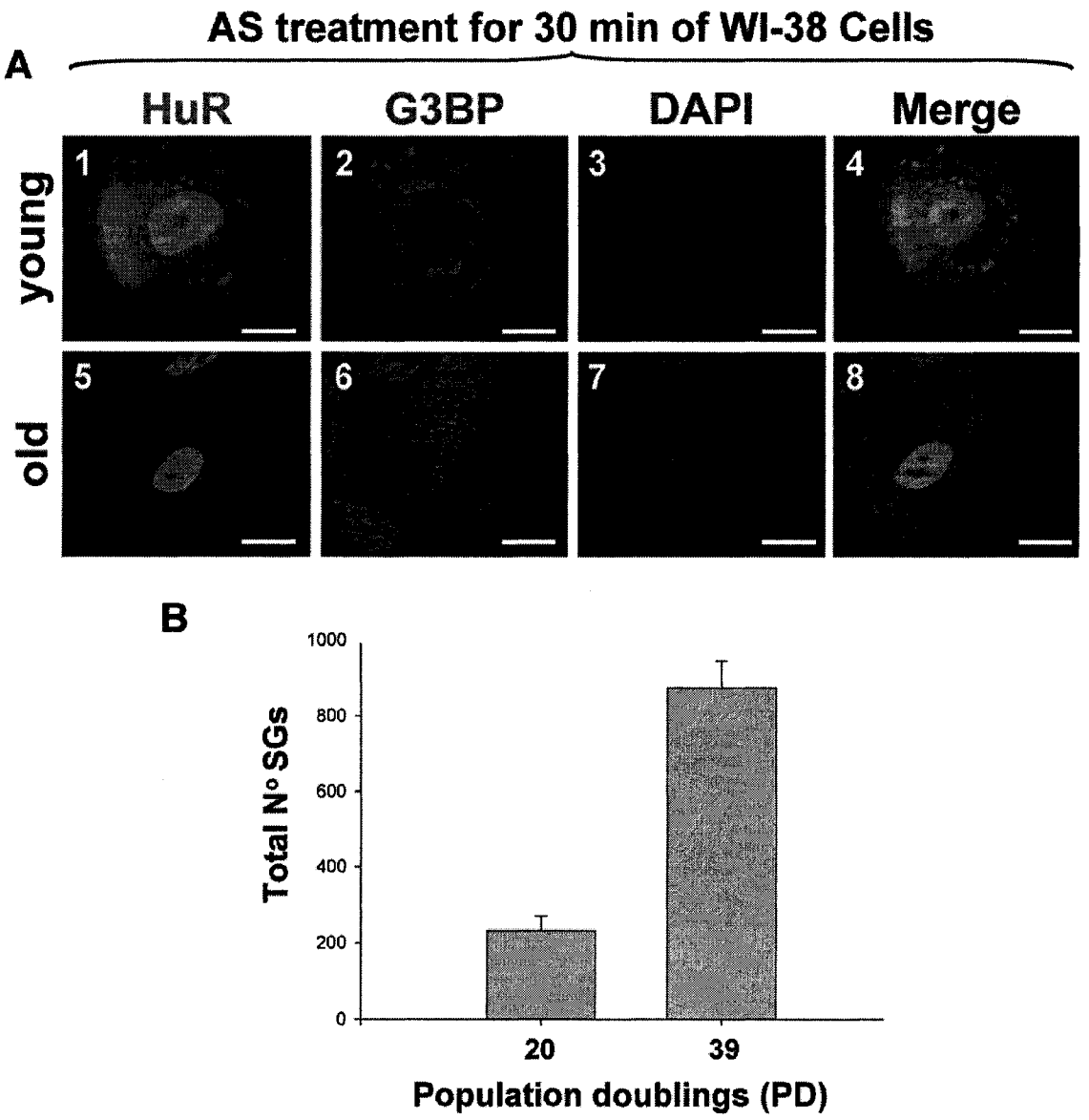


Figure 3. Oxidative stress induces the formation of stress granules in WI38 cells. (A) After being treated with arsenite (0.5mM) for 30 min., proliferative (PD=20) and senescent cells (PD=39) were subjected to immunofluorescence with antibodies specific to HuR and G3BP. (B) The graph illustrates the number of stress granules formed (as evidenced by G3BP staining) on the different days of senescence. The number of stress granules in three random fields with 10 random cells was counted and the average calculated.

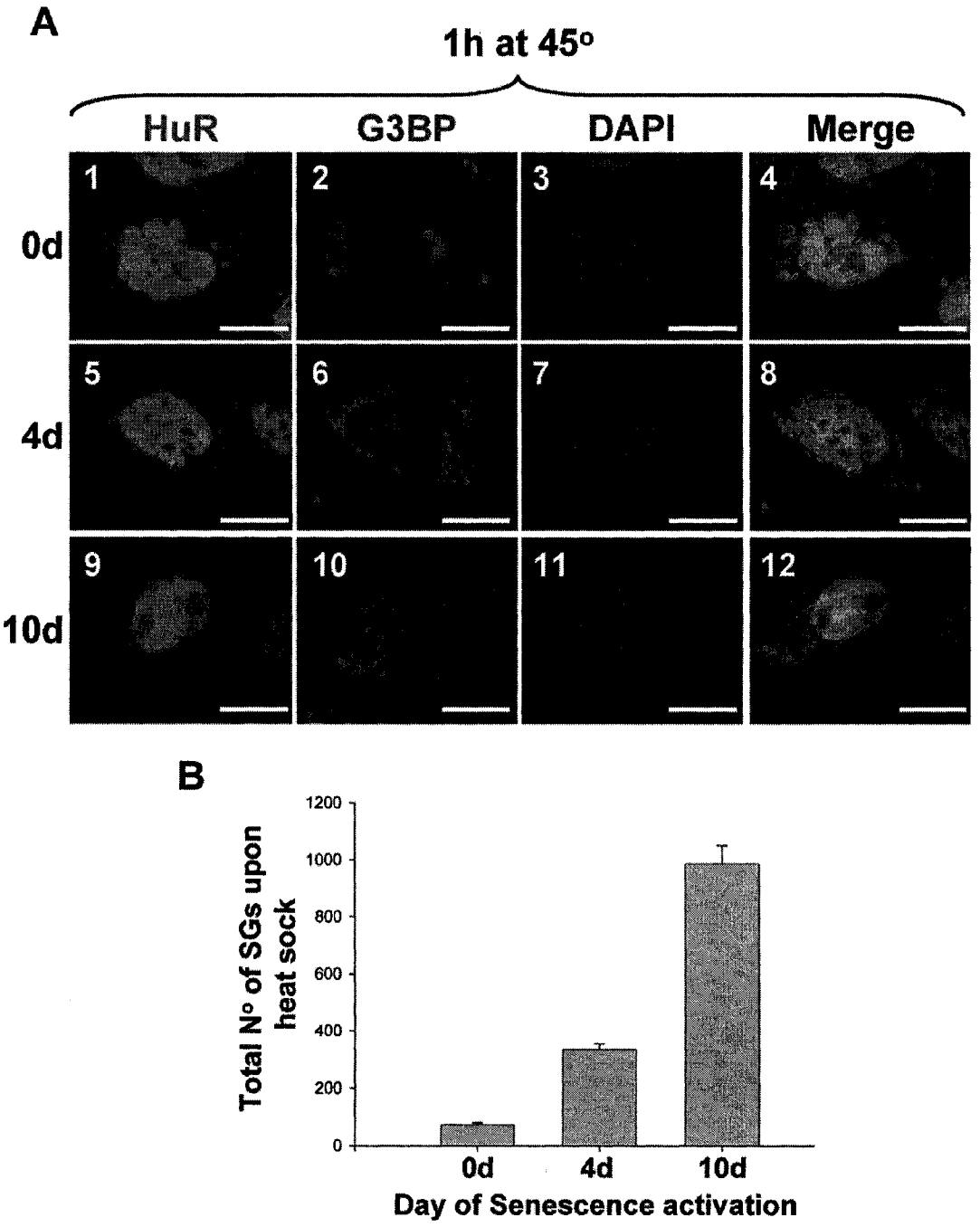


Figure 4. Heat shock induces the formation of stress granules in IDH4 cells. Proliferative and senescent cells were treated for at 45 °C for 1h. Cells were subsequently fixed, permeabilized and analyzed by immunofluorescence with antibodies against two surrogate markers of SGs: HuR and G3BP. The graph illustrates the number of stress granules formed on the different days of senescence. The number of stress granules in three random fields with 10 random cells was counted and the average calculated.

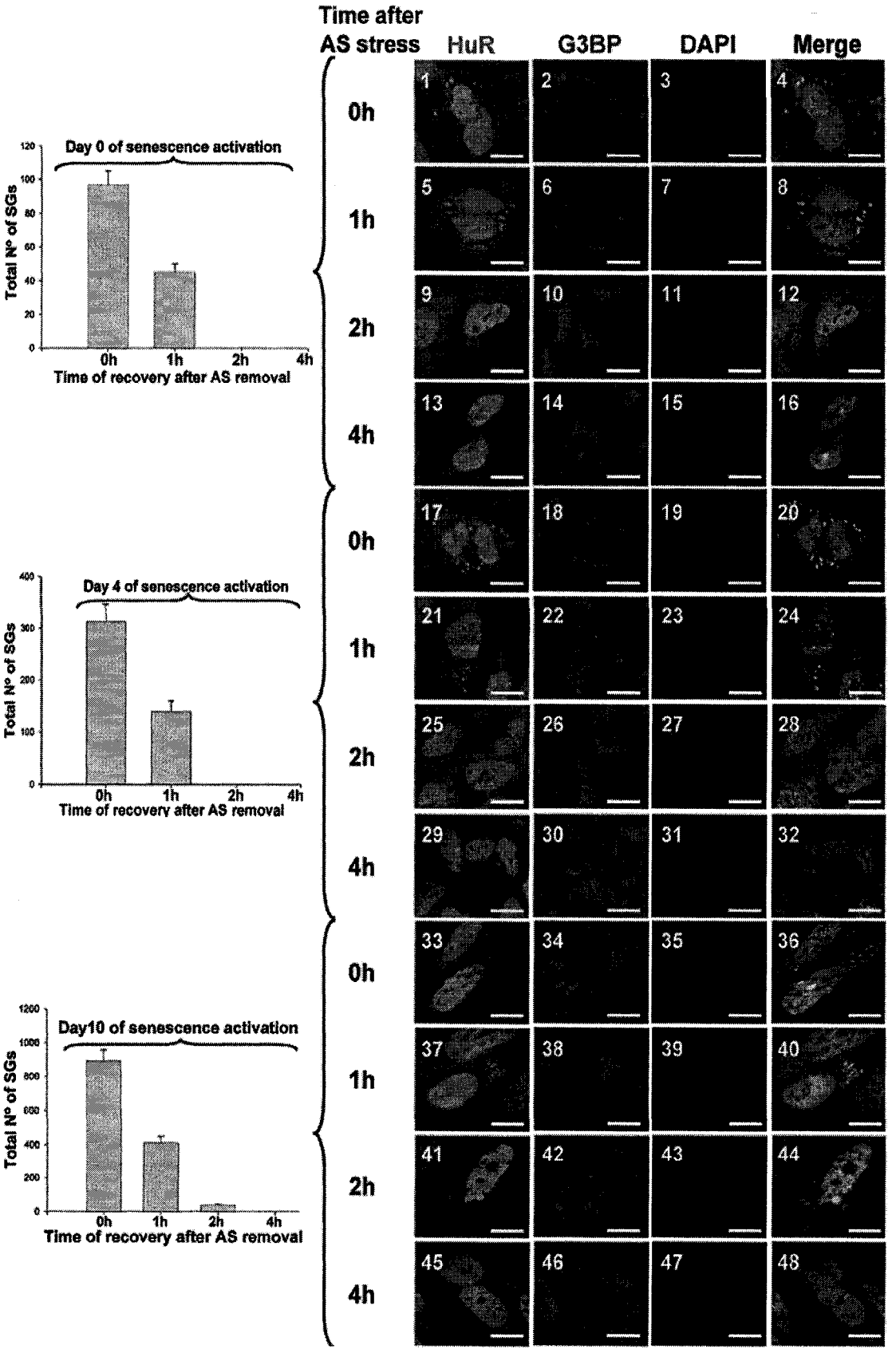
senescent cells from arsenite induced oxidative stress. AS-treated proliferative and senescent IDH4 cells were switched to AS-free media and incubated at 37° C for several hours. Cells were harvested at different time points during the recovery process and used for western blot analysis with anti-Hsp70 and -G3BP antibodies. As expected a small delay in the expression of Hsp70 protein was observed in late senescent cells (day 10) compared to their proliferative (day 0) and early senescent counterparts (day 4) (Figure 6). Hence, together these results indicated that the slow recovery rate observed in senescent cells could be explained in part by the high number of SGs as well as the delay in the expression of Hsp70 protein.

Despite a general effect on protein translation, AS treatment specifically affected the expression of p21 protein in senescent cells

It is well established that in proliferative cells, the formation of AS-induced SGs correlates with the phosphorylation of the translation initiation factor eIF2α (Kedersha et al., 1999). To assess whether this is also the case in senescent cells, IDH4 cells grown in the presence (proliferative) or absence (senescent) of dexamethasone and exposed or not to 0.5 mM AS for 30 min, were harvested at different days of the senescence process. Total extracts prepared from these cells were subjected to western blot analysis using antibodies against the phosphorylation (p-eIF2α) and the wild-type (wt- eIF2α) isoforms of eIF2α protein. As expected, the p-eIF2α isoform was detected at all stages of the senescence process only in AS-treated cells (Figure 7A, compare lanes 1-5 to 6-10). Our experiments showed that despite the general decrease in the expression levels of wt-eIF2α

protein in late senescence cells, the p-eIF2α isoform was still visible (Figure 7A, compare lanes 8-9

Figure 5. Stress granules disassemble during the recovery of both proliferative and senescent cells from arsenite induced oxidative stress. . Proliferative and senescent IDH4 cells were incubated with arsenite (0.5mM) for 30 min. Cells were subsequently washed twice with PBS, replenished with fresh media and incubated for various periods of time at 37°C. Cells were subsequently fixed, permeabilized and analyzed by immunofluorescence with antibodies against two surrogate markers of SGs: HuR and G3BP. The graph illustrates the number of stress granules during the recovery of both proliferative and senescent IDH4 cells. The number of stress granules in three random fields with 10 random cells was counted and the average calculated.


Time after AS stress
HuR
G3BP
DAPI
Merge
Day 0 of senescence activation
Total N° of SGs
Time of recovery after AS removal
0h
1h
2h
4h
Day 4 of senescence activation
Day10 of senescence activation

to 4-5). The presence of p-eIF2α is not only a marker of cell response to AS treatment, but is also an indication of a dramatic decrease in general mRNA translation. This decrease is one of the major consequences of cell response to a variety of stresses (Holcik and Sonenberg, 2005). To assess the effect of AS on the translation process in senescent cells, IDH4 treated as described above (+/- dexamethasone/AS) were incubated for 30 min with [^{35}S]methionine and the synthesis of de novo proteins was determined using a 10% SDS-polyacrylamide gel (Figure 7B). We showed that AS treatment negatively affected general translation in both proliferative and senescent cells (Figure 7B). Together these results showed that, as in other cell systems (Kedersha et al., 1999), the assembly of As-induced SGs correlated with a general decrease of protein synthesis.

The development and the maintenance of the senescence phenotype require the expression of several factors such as p53, CUGBP1 and p21 (Collado et al., 2007; Iakova et al., 2004). To examine the expression of these proteins in senescent cells exposed to AS, we prepared total cell extracts as described in Figure 7A and performed a western blot analysis using specific antibodies (Figure 7C). Although, at early senescence (days 2 to 4), AS did not affect the expression of any of these factors, it triggered a significant reduction (>~75%) in the levels of only p21 protein (Figures 7C, compare day 0-4 to 8-10 and Figure 7D). Our results indicated that, despite the high expression levels of p53, which is known as one of the main activator of p21 transcription during senescence (el-Deiry et al., 1994; el-Deiry et al., 1993), AS stress significantly reduced p21 expression. However, we do not know whether this affects the mechanism by which AS exercises this effect.

Figure 6. The expression of Hsp70 increases during the recovery of proliferative (A), early senescent (B) and late senescent (C) IDH4 cells from arsenite induced cells. Proliferative and senescent IDH4 cells were incubated with arsenite (0.5mM) for 30 min. Cells were subsequently washed twice with PBS, replenished with fresh media and incubated for various periods of time at 37°C. Western blots, performed with total cell extracts harvested at various time points post recovery, were analyzed with antibodies specific to Hsp70 and G3BP (used as the loading control).

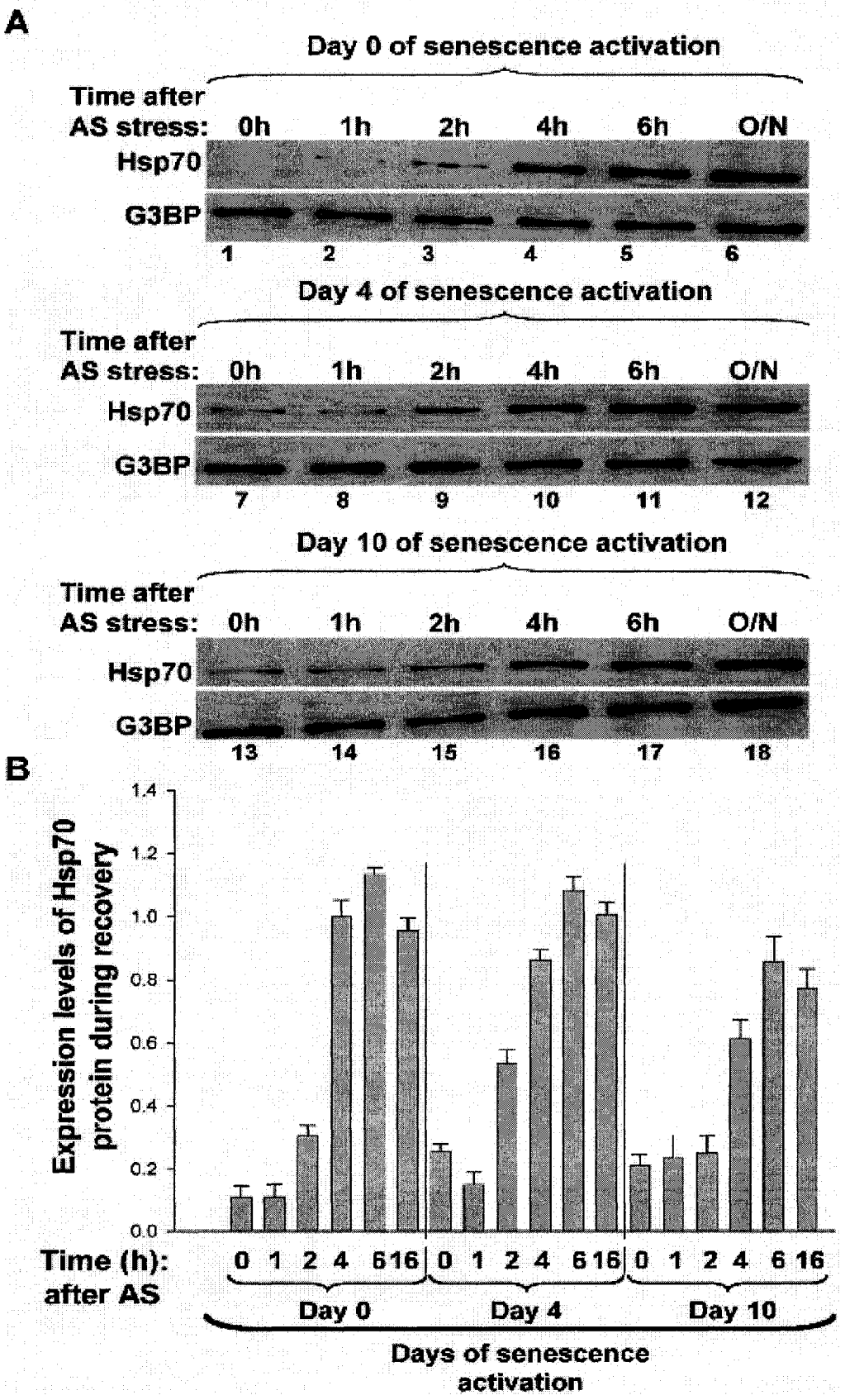
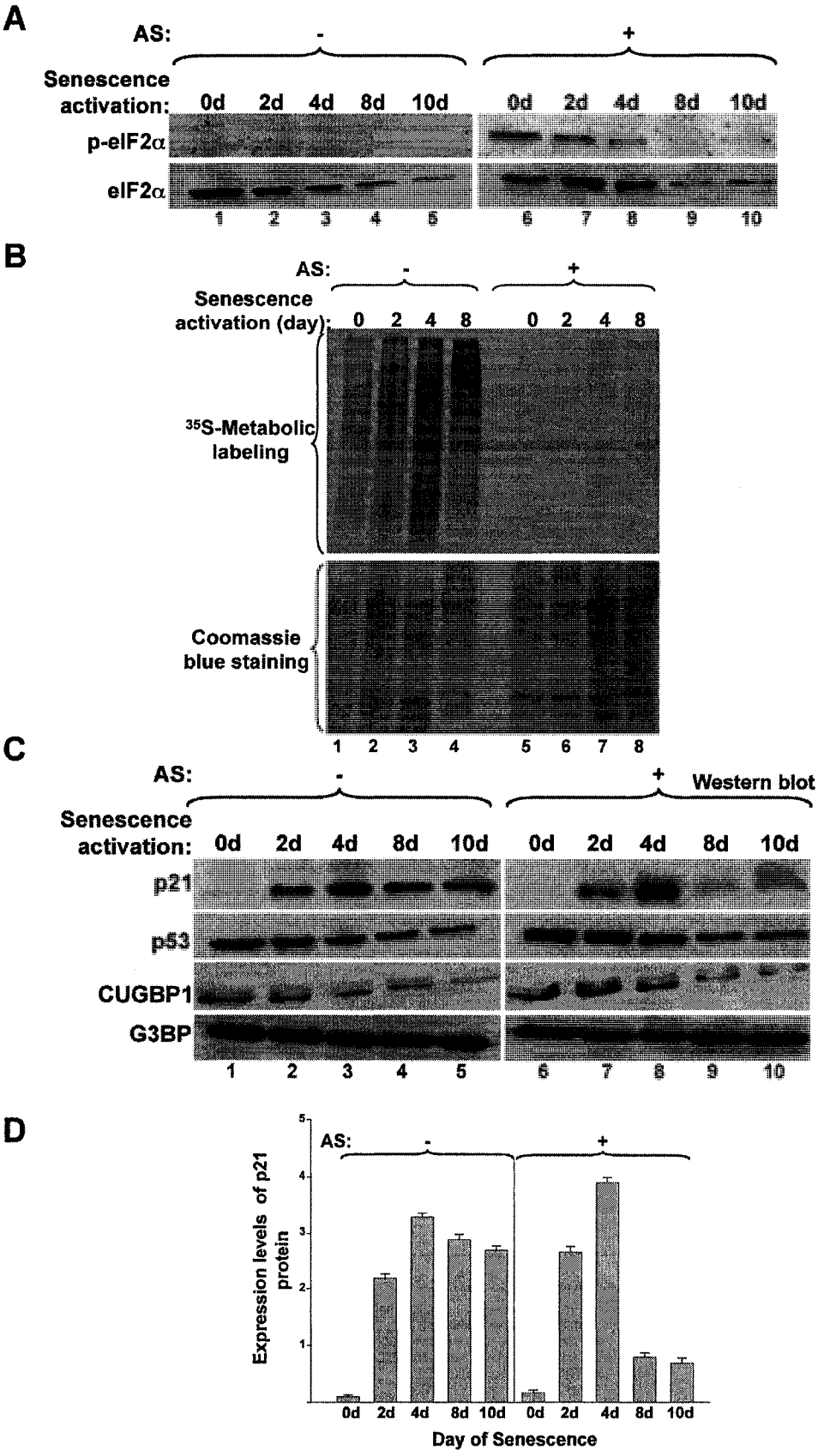

A
Day 0 of senescence activation
Time after AS stress:
0h
1h
2h
4h
6h
O/N
Hsp70
G3BP
1
2
3
4
5
6
Day 4 of senescence activation
Time after AS stress:
0h
1h
2h
4h
6h
O/N
Hsp70
G3BP
7
8
9
10
11
12
Day 10 of senescence activation
Time after AS stress:
0h
1h
2h
4h
6h
O/N
Hsp70
G3BP
13
14
15
16
17
18
B
Expression levels of Hsp70 protein during recovery
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Time (h): after AS
0 1 2 4 6 16 0 1 2 4 6 16 0 1 2 4 6 16
Day 0
Day 4
Day 10
Days of senescence activation

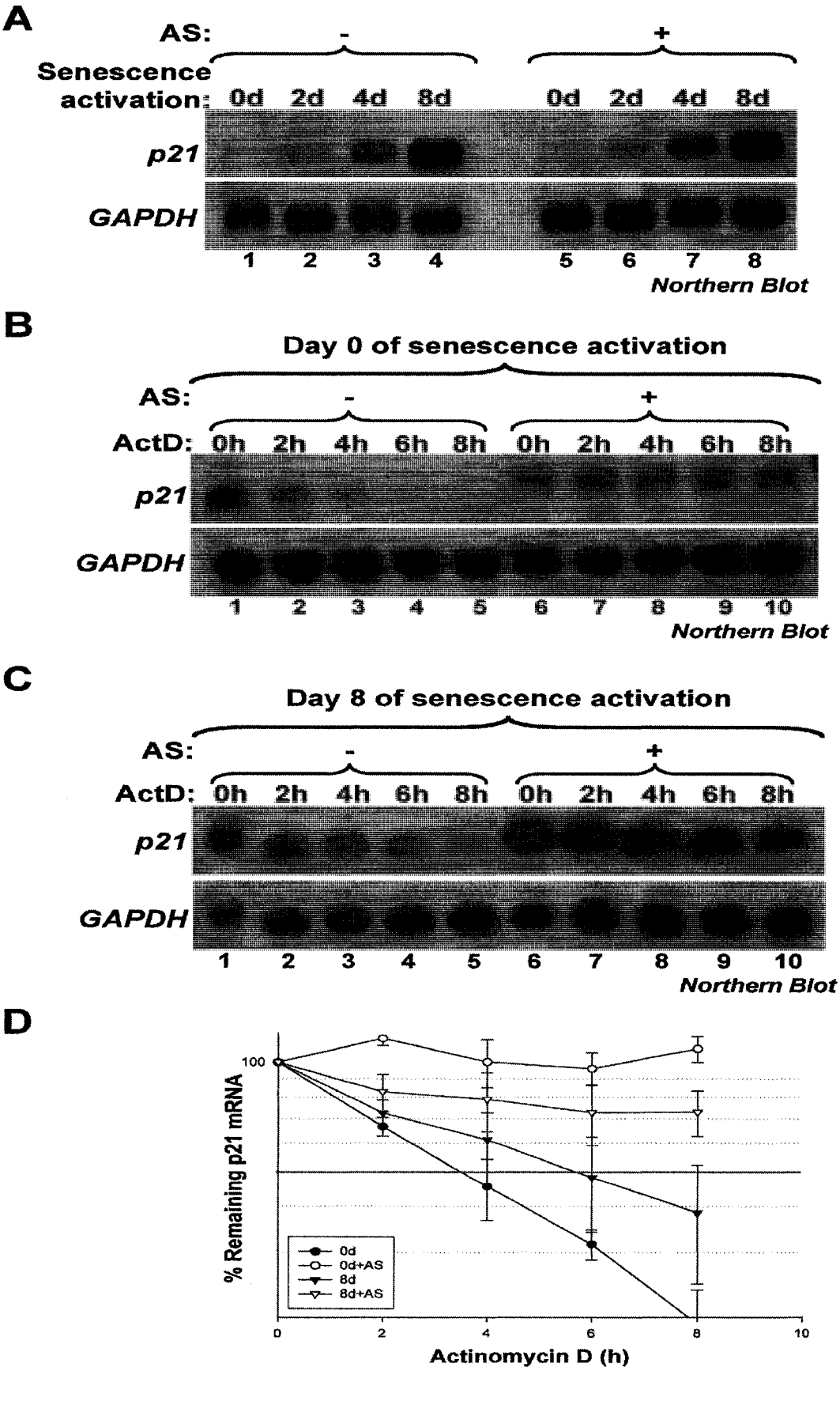
Figure 7. Expression of proteins in senescence
(A) Expression of eIF2α and eIF2α phosphorylation during senescence. Western blots were performed with cell extracts harvested at different time points during the induction of senescence in the presence or absence of arsenite. Blots were subsequently probed with antibodies specific to p-eIF2α and eIF2α. (B) General protein translation is inhibited in arsenite treated IDH4 cells. Cells were incubated with arsenite (0.5 mM) for 30 min. and then incubated with [^{35}S]methionine (50 μCi/ml) for another 30 min. Proteins were resolved by SDS-polyacrylamide gel and stained with Coomassie blue, followed by detection by autoradiography. (C) Arsenite induced oxidative stress affects the expression of p21 in senescent cells. Western blots were performed as described in (A) and probed with antibodies to p21, p53, CUGBP1 and G3BP. G3BP levels were used as a loading control.


A
AS:
-
+
Senescence activation:
0d 2d 4d 8d 10d
0d 2d 4d 8d 10d
p-eIF2α
eIF2α
1 2 3 4 5 6 7 8 9 10
B
AS:
-
+
Senescence activation (day):
0 2 4 8
0 2 4 8
35S-Metabolic labeling
Coomassie blue staining
1 2 3 4 5 6 7 8
C
AS:
-
+
Western blot
Senescence activation:
0d 2d 4d 8d 10d
0d 2d 4d 8d 10d
p21
p53
CUGBP1
G3BP
1 2 3 4 5 6 7 8 9 10
D
AS:
-
+
Expression levels of p21 protein
5
4
3
2
1
0d 2d 4d 8d 10d 0d 2d 4d 8d 10d
Day of Senescence

AS treatment prevents the translation of p21 mRNA in late senescence cells by triggering its rapid recruitment to SGs

We were surprised to observe that p53 expression was high in both proliferative and sensescent cells (Figure 7C). However, this result was consistent with the fact that the large T antigen [which is induced by dexamethasone to prevent the induction of senescence in IDH4 (Wright et al, 1989)] has been previously shown to stabilize and probably inactivate p53 (Hsieh et al.and, 2000; Deppert et al., 1987; Borger and DeCaprio, 2006). Hence, AS could have affected the expression of the p21 protein in senescent cells by altering its p53-mediated transcription, stability or the translation of its mRNA. To assess these possibilities we first performed northern blots analysis on total mRNA isolated from proliferative and senescent IDH4 cells exposed or not to 0.5 mM AS for 30 min. We observed that as expected, (Choudhury et al., 2007; Ju et al., 2007) the steady state levels of p21 mRNA significantly increased during the senescence process in the presence or absence of AS (Figure 8A). Next, we performed Actinomycin D pulse chase experiments to examine the effect of AS treatment on the stability of p21 mRNA in IDH4 cells grown in the presence (day 0) or absence of dexamethasone (day 8) (Figures 8B-C). As with other stresses (Mazroui et al., 2007) AS treatment increased the half-live of p21 mRNA from ~3.5h to >10h in proliferative and from ~5.5h to >10h in late senescent cells (Figure 8D). Together, our data showed that the pronounced decrease in the expression of p21 protein is not due to changes in the stability or the steady state levels of the p21 message. Hence, it is possible that exposing senescent cells to AS stress triggers cellular mechanisms that interfere with the translation of p21 message.

Figure 8. p21 mRNA expression and stability in senescence
(A) p21 mRNA steady state levels increase during senescence either with or without treatment of arsenite. Total mRNA was harvested at different periods of time during senescence in the presence or absence of arsenite p21 mRNA levels were subsequently verified by Northern blot analysis. GAPDH mRNA was assessed as a loading control. (B) and (C) Oxidative stress stabilizes p21 mRNA in both proliferative and senescent IDH4 cells. Proliferative and senescent IDH4 cells were treated with or without arsenite and then incubated with 5 μg/ml actinomycin D (ActD) for the indicated times. Total RNA was prepared and p21 mRNA levels were subsequently verified by Northern blot analysis. GAPDH mRNA was assessed as a loading control. Northern blots were performed using 25 μg of total RNA from proliferative cells and 15 μg of total RNA from senescent cells. (D) The half life of p21 mRNA increases during senescence in the presence of stress. p21 mRNA was quantified using the ImageQuant software program, standardized against GAPDH message and plotted as the percentage of remaining mRNA compared with message levels at the 0 time point (where there is 100% maximum mRNA level).

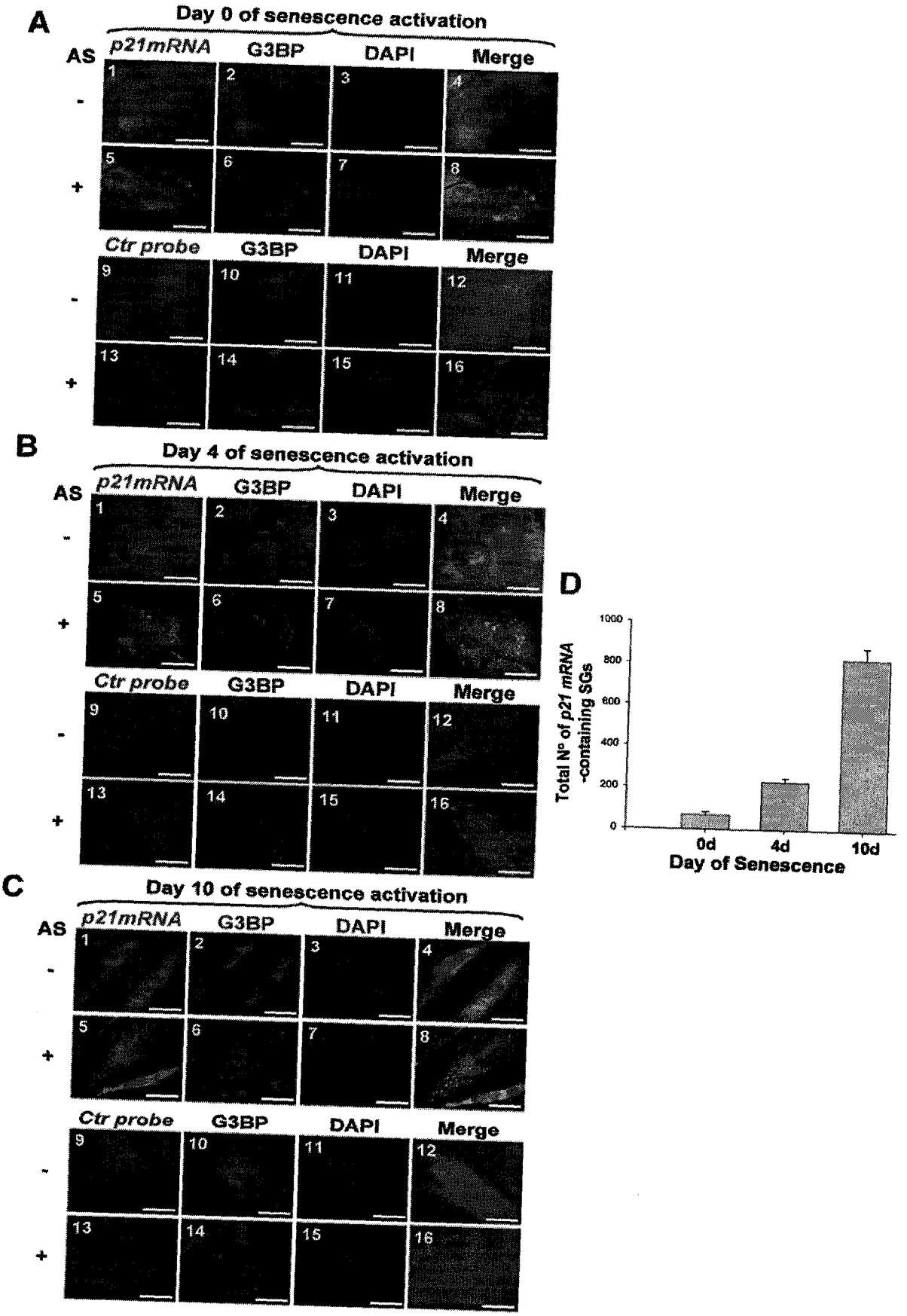

A
AS:
-
+
Senescence activation:
0d 2d 4d 8d
0d 2d 4d 8d
p21
GAPDH
1 2 3 4 5 6 7 8
Northern Blot
B
Day 0 of senescence activation
AS:
-
+
ActD:
0h 2h 4h 6h 8h
0h 2h 4h 6h 8h
p21
GAPDH
1 2 3 4 5 6 7 8 9 10
Northern Blot
C
Day 8 of senescence activation
AS:
-
+
ActD:
0h 2h 4h 6h 8h
0h 2h 4h 6h 8h
p21
GAPDH
1 2 3 4 5 6 7 8 9 10
Northern Blot
D
% Remaining p21 mRNA
100
0d
0d+AS
8d
8d+AS
0 2 4 6 8 10
Actinomycin D (h)

We have previously reported that treating cells with MG132 for a few hours stabilized p21 message and reduced its translation by triggering its rapid recruitment to SGs (Mazroui et al., 2007). RNA FISH (Fluorescence In Situ Hybridization) experiments as described (Mazroui et al., 2007), were performed on IDH4 cells induced or not for senescence in the presence or absence of AS using an anti-sense DIG-labeled RNA probe specific to p21 message. We observed that endogenous p21 mRNA was recruited to AS-induced SGs in proliferative, early and late senescent cells (Figure 9A-D). Under these conditions, the number of p21 mRNA-containing SGs significantly increased as the cells enter senescence (Figure 9D) at almost the same levels as those containing G3BP (Figure 9). Therefore, these data indicated that the recruitment of p21 mRNA to stress granules may explain the decreased p21 protein levels seen in stressed senescent cells.

The RNA binding protein CUGBP1, a translational regulator of p21 mRNA, is recruited to arsenite induced stress granules in both proliferative and senescent IDH4 cells

Several RNA-binding proteins have been demonstrated to play a key role in the translation of p21 mRNA. In young (proliferative) cells, the RNA-binding protein calreticulin binds to the 5' region of the p21 mRNA inhibiting its translation. CUGBP1, another RNA binding protein, has been shown to displace calreticulin from the p21 mRNA and thus enhancing its translation (Iakova et al., 2004) leading to senescence. Therefore, we asked whether the expression and cellular localization of CUGBP1 change

Figure 9. p21 mRNA is recruited to stress granules in arsenite treated proliferative and senescent cells. Proliferative and senescent IDH4 cells were incubated with arsenite (0.5 mM) for 30 min. The cells were then fixed, permeabilized and incubated with DIG-labeled *in vitro* transcribed antisense probe to detect p21 mRNA and with sense RNA probe as a control (Ctr probe, panels 9–16). Detection of G3BP with anti-G3BP antibodies enabled the visualization of stress granules. The number of stress granules containing p21 mRNA was counted for 10 random cells in a field and three random fields were used and the average number per field was calculated. The total average number of stress granules was plotted for each day of senescence.


A
Day 0 of senescence activation
AS
p21mRNA
G3BP
DAPI
Merge
Ctr probe
B
Day 4 of senescence activation
C
Day 10 of senescence activation
D
Total N° of p21 mRNA -containing SGs
1000
800
600
400
200
0
0d
4d
10d
Day of Senescence

during senescence when the cells were stressed with arsenite. We performed immunofluorescence experiments with an anti-CUGBP1 antibody to test its presence in stress granules. We demonstrated that CUGBP1 is recruited to stress granules during senescence when the cells were exposed to 0.5 mM AS for 30 min (Figure 10). Furthermore, as shown with other experiments (Figures 2 and 9) the localization of CUGBP1 to AS-induced SGs increased considerably during senescence (Figure 10). Together, our data indicated that AS-mediated CUGBP1 recruitment to SGs is part of the cellular mechanisms that block the translation of p21 message in senescent cells exposed to oxidative stress.

The translation of p21 increases in senescent cells during the recovery from AS-induced stress

In order to verify how the recovery from stress will affect the translation of p21 message, western blot analyses were performed with total extracts from senescent cells harvested at different time points after the recovery from arsenite induced oxidative stress. We showed that p21 protein levels significantly increased 2 to 4h after the removal of AS stress (Figure 11A-B). This increase coincided with the disappearance of stress granules in the early and late senescent cells (See Figure 5). During the recovery from stress, both CUGBP1 and the p21 mRNA are released from granules, thus likely enabling the CUGBP1 mediated translation of the p21 mRNA. Together, our data here showed that the recovery of p21 translation correlates with the disassembly of stress granules.

Figure 10. CUGBP1 was recruited to stress granules. IDH4 cells at various time points (0d. 4d, 10d) after the induction of senescence were treated for 30 minutes with or without 0.5mM arsenite. Cells were subsequently fixed, permeabilized and were analyzed by immunofluorescence with antibodies against CUGBP1 and G3BP. The graph illustrates the number of stress granules formed (as evidenced by CUGBP1 staining) on the different days of senescence.
The number of stress granules in three random fields with 10 random cells was counted and the average calculated.

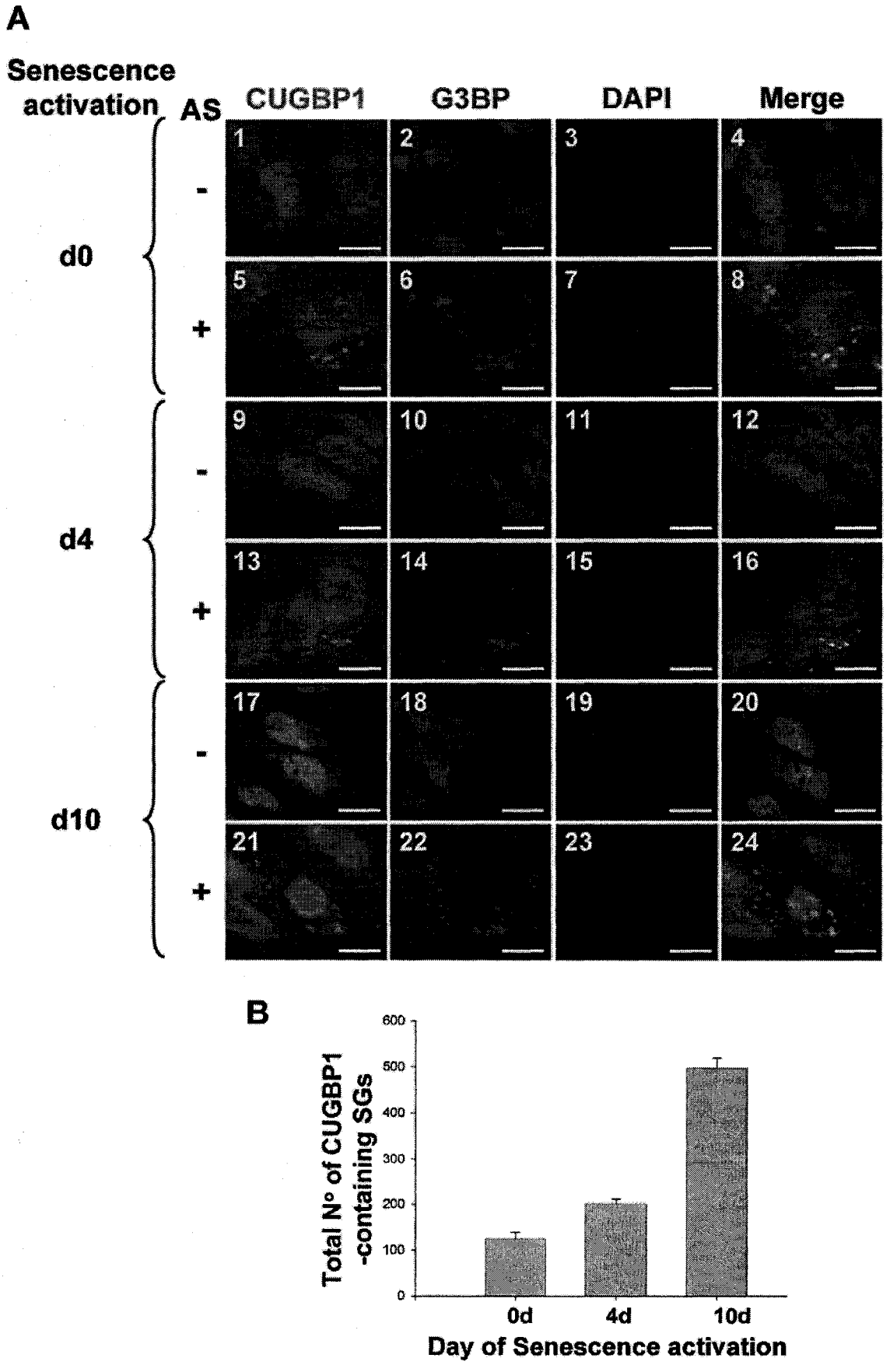
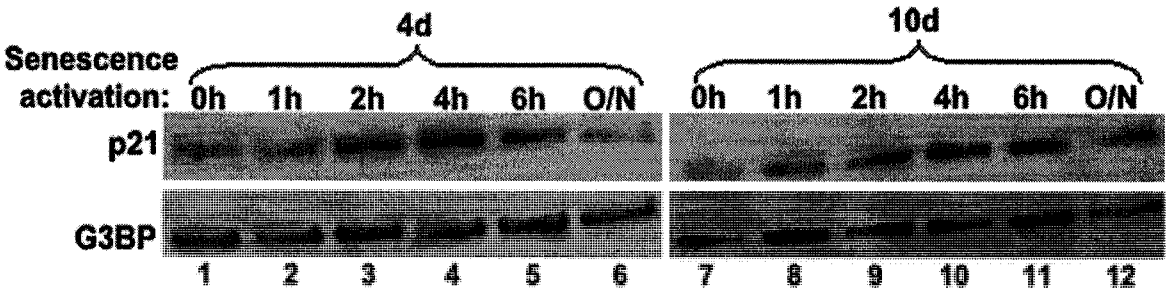

A
Senescence activation
AS
CUGBP1
G3BP
DAPI
Merge
d0
d4
d10
-
+
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
B
Total N° of CUGBP1 -containing SGs
600
500
400
300
200
100
0
0d
4d
10d
Day of Senescence activation

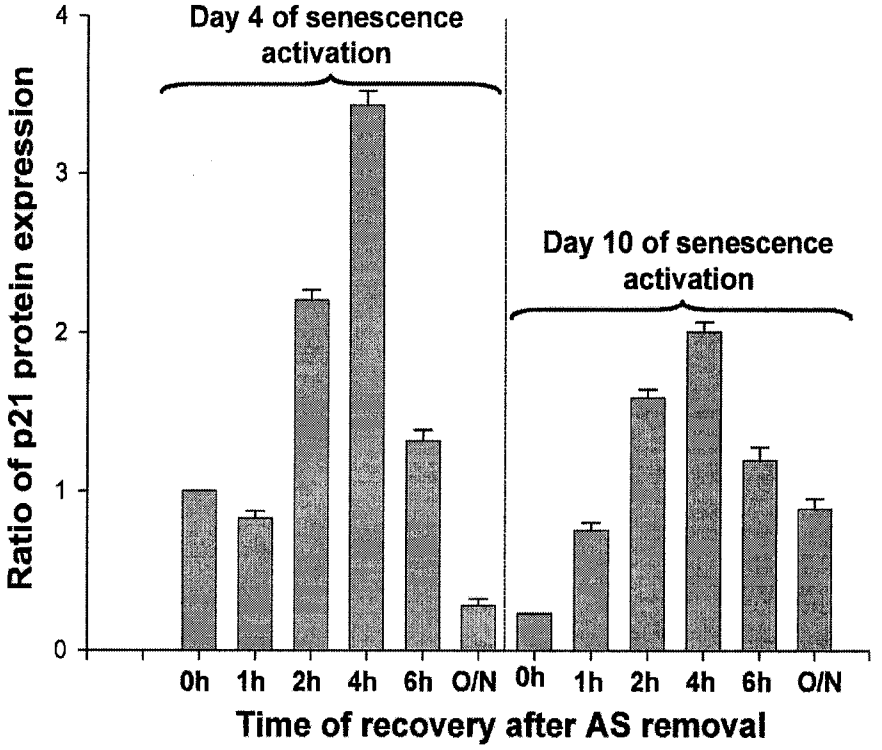
Figure 11. Recovery of p21 translation after removal of stress
(A) p21 proteins levels increase during the recovery of senescent cells from stress. Early (4d) and late (10d) senescent IDH4 cells were incubated with arsenite (0.5 mM) for 30 min. Cells were subsequently washed twice with PBS, replenished with fresh media and incubated for various periods of time at 37°C. Western blots, performed with total cell extracts harvested at various time points post recovery, were analyzed with antibodies specific to p21 and G3BP (used as the loading control). (B) Graph illustrating the quantified p21 protein levels in (A) normalized against G3BP loading control.

A



Western blot

B



Materials and methods

Cell lines and cultures

IDH4 cells were generously provided by Dr. Myriam Gorospe. WI38 cells were generously provided by Dr. Chantal Autexier. All cell lines were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS; Sigma) and penicillin/ streptomycin (Sigma). Media supplemented with 1 μM of dexamethasone (needed for the induction of the SV40 large T antigen) was used to ensure proliferation of IDH4 cells, (Wright et al., 1989). In order to induce the senescence of IDH4 cells, dexamethasone was removed from the media and FBS was replaced by charcoal-stripped FBS (Sigma). Serial passage of cells was performed to induce the senescence of WI38 cells.

Reagents

Sodium m-Arsenite, Actinomycin D and cycloheximide were purchased from Sigma.

Antibodies

Anti-p16, anti-CUGBP1 and anti-p21 antibodies were obtained from Santa Cruz Biotechnology. Anti-eIF2α and anti-phosho-eIF2α antibodies were obtained from Cell Signaling. Anti-p53 antibody was kindly provided by Dr. P. Branton (McGill University, Canada). Anti-HuR and anti-G3BP antibodies were previously described (Gallouzi et al., 2000; Mazroui et al., 2006). Secondary antibodies were obtained from Biocan Jackson.

β-galactosidase staining

The Senescent Cells Staining Kit (obtained from Sigma) was used as to detect senescent cells as previously described (reference?) Briefly cells were grown on 35 mm plates. The cells were washed twice with 1X PBS and fixed with 1X fixation buffer. After washing

the cells three times with 1X PBS, 1 ml of staining solution was added to the cells and incubated overnight at 37°C.

Immunofluorescence

Cells were grown to confluency on coverslips. The cells were washed with then washed with PBS and fixed with 3% paraformaldehyde. The cells were subsequently permeabilized with 0.1% Triton X-100/PBS, and washed three times with cold 1% normal goat serum/PBS. The, coverslips were incubated with primary antibody diluted in 1% normal goat serum/PBS for 1h at room temperature. Coverslips were then washed twice with 1% normal goat serum/PBS and incubated with secondary antibody coupled to Alexa Fluor488/594 (Molecular Probes) for 1h at room temperature. DAPI (1:20000) was added 5-10 min prior to the end of the incubation with the secondary antibodies. Finally, coverslips were washed twice with PBS and once with H_2O. Vector Shield (Vector Lab) was put on the slides and coverslips were sealed using nail polish. Fluorescence microscopy was performed using the Zeiss Axiovision 4.3 microscope.

Preparation of cell extracts and Western Blot

Cells were washed twice with cold PBS, scraped then centrifuged at 3000rpm for 5 min. The cell pellet was subsequently lysed in lysis buffer (50 mM HEPES, pH=7.0, 150 mM NaCl, 10% Glycerol, 1% Triton, 10 mM pyrophosphate sodium, 100 mM NaF, 1 mM EGTA, 1.5 mM MgCl2, 1X protease inhibitor, 1 mM orthovanadate and 0.1 mM PMSF). Protein concentrations were determined by Bradford assay. The Laemmli loading dye was added to a final concentration of 1X. The extract were heated at 95°C prior to loading on a 10% resolving SDS-polyacrylamide gel. Gels were transferred to nitrocellulose membrane and probed with antibodies.

Northern Blot Analysis of mRNA

Northern blots were performed as previously reported (Di Marco et al., 2005). Total RNA was extracted from cells using the TRIzol reagent (Invitrogen). The RNA was transferred to a Hybond-N membrane, hybridized with [^{32}P]dCTP-labeled human p21 and GAPDH probes, washed and then exposed to Biomax films.

FISH (Fluorescence In Situ Hybridization)

The DNA fragment of ~ 500 bp corresponding to the coding region of human p21 was amplified by PCR using the following primers fused to either a T7 or T3 minimal promoter sequence: T7-p21 Forward: 5'-TAA TAC GAC TCA CTA TAG GGG AAG GTA GCT GGC ATG AAG CC-3' and T3-p21 reverse: 5'-AAT TAA CCC TCA CTA AAG GGG AAG ACC ATG TGG ACC TGT CA-3' The PCR product was used as the template for in *vitro transcription* of the p21 probe needed for the FISH. The antisense (T3) and sense (T7) probe was prepared using DIG RNA Labeling Mix from Roche Diagnostics. The RNA probe was quantified, denatured and incubated with permeabilized cells at 37°C overnight in the hybridization buffer (50% formamide, 5X SSC, 50 mM phosphate buffer, pH=7.4, 5X Denhardt's, 1 mM EDTA and 250 ng/µl of salmon sperm DNA). After the hybridization, the cells were incubated with anti-G3BP antibody for 1h at room temperature. Finally, the cells were incubated with secondary goat anti-rabbit antibody and anti-DIG antibody for immunofluorescence.

Discussion

Many studies have shown that stress granules can be induced under different stresses in proliferative cells. However, no reports are available to date about the induction of stress granules in senescent cells. We do not know how these cytoplasmic foci, if any, mediate the response of senescent cells to environmental stress. In this study, we show that stress granules can be induced in senescent cells and their number significantly increases with the process of senescence. The assembly of these granules correlates with the phosphorylation of eIF2α as well as a dramatic decrease in general translation in both proliferative and senescent cells. We also show that the recruitment of both the p21 message and CUGBP1 in stress granules leads to a significant decrease in the expression of p21 protein in senescence. Moreover, senescent cells recover more slowly than young cells in terms of disassembly of stress granules and the expression of Hsp70 protein.

Despite the fact that their ability to divide or to differentiate is impaired, senescent cells are still able to adapt to different growth conditions. However, their adaptive capacity to environmental stresses decreases (Helenius et al., 1999; Bruunsgaard and Pedersen, 2000). The formation of stress granules is a common protection mechanism for cells by which general translation of proteins is blocked (Kim et al., 2005) and untranslated mRNAs are stored to allow a quick recovery when the stress is removed. Our data show that this is also the case for senescent cells, where stress granules can be induced in response to different stresses (Figure 2, panels 29-32). As a result, general translation of proteins during senescence is inhibited when cells are exposed to stresses such as AS treatment (Figure 7B). Moreover, the number of stress granules increases considerably with senescence (Figure 2, graph). This result is consistent with the concept that one of

the consequences of the irreversible cell cycle arrest state is a decreased ability to respond to a variety of stresses. Therefore, unlike proliferative cells, it is possible that to cope with extracellular assaults senescent cells have to form more stress granules. Since the induction of stress granules depends on the phosphorylation of eukaryotic translation initiation factor eIF2α (Kimball, et al. 2003), we investigated the expression of the phosphorylated and unphosphorylated forms of eIF2α during cellular senescence either with or without treatment of AS. Our data show that although the expression of eIF2α decreases during senescence, AS induces the phosphorylation of eIF2α which results in the formation of stress granules (Figure 7A). The data support previous studies on the expression of eIF2α and p-eIF2α in different aged rat tissues (Hussain and Ramaiah, 2007). Of note we observed that although the general levels of eIF2α decrease (Figure 7A), the ratio between p-eIF2α/eIF2α is significantly lower in senescent cells compared to their proliferative or early senescent counterparts (data not shown). It is not clear why the levels of p-eIF2α decrease despite the significant increase in the number of SGs in senescent cells. It is possible that the cellular mechanisms responsible for triggering AS-mediated SGs assembly switches from the p-eIF2α-dependent in proliferative cells to p-eIF2α-independent mechanism in senescent cells. It will be of high interest to further investigate this possibility.

It is known that senescent cells recover from stresses more slowly than proliferative cells (Zarzhevsky et al., 2001; Rosenfeldt et al., 2004; Honda and Matsuo, 1987; Romani et al., 1968). Our data support the results of those studies. We show that the disassembly of stress granules occurs at 2h after removal of AS for proliferative and early senescent (4d of senescence) cells (Figure 5, panels 9-12, 25-28). However, the disassembly is delayed until 4h for late senescent cells (10d of senescence) (Figure 5,

panels 45-48). Previous studies have shown that Hsp70 plays a key role in the disassembly of SGs (Mazroui et.al.) and is involved in the recovery of translation (Van Nieuwenhoven et al., 2001; Liu et al., 1992; Rylander et al, 2005). We observed that this could be the case in senescent cells. We showed that the expression of Hsp70 recovers more slowly in senescent (4h) when compared to proliferative (2h) cells after the removal of AS which coincides with the disassembly of stress granules. However, it is still unclear how Hsp70 protein triggers SGs disassembly.

p21 is a well studied protein involved in senescence (Stein et al., 1999). Therefore, we investigated the effect of the formation of stress granules on the expression of this protein. We observed that the expression of p21 protein increases during early senescence to a maximum level in both stressed and unstressed cells (Figure 7C) and then decreases and remains unchanged at late senescent. However, the level of p21 is considerably decreased in late senescence cells exposed to AS stress (Figure 7C-D). Its transcription factor, p53, does not undergo significant decrease during senescence (Figure 7C) and p21 mRNA level increases during senescence (Figure 8A) . This indicates that the variation in p21 levels in stressed cells is unlikely to be the result on the transcription activity of its gene.

Under stress, p21 mRNA has been shown to be recruited to stress granules where it is stabilized (Mazroui et al., 2007). Therefore, we hypothesize that p21 mRNA is recruited to stress granules during senescence, leading to inhibition of its translation and decreased protein level. Our data support this hypothesis and showed that p21 mRNA localizes in stress granules in senescence cells exposed to AS treatment (Figure 9). The number of SGs containing p21 mRNA increases significantly during senescence (Figure 9), which

correlates with the decrease in its protein levels. Since the transcription and stability of mRNA also affect the expression of proteins, we investigated their effect on the p21 protein levels. Our data show that the decrease in p21 protein is not due to the changes in the transcription of p21 mRNA since the steady state levels increased during senescence for either stressed or unstressed cells (Figure 8A). Furthermore, this effect is also not due to changes in the stability of the message. Indeed, p21 mRNA is stabilized in proliferative and senescent cells treated with 0.5 mM AS for 30 min (Figure 8B-C). Our data show that under these stress conditions, the half-life of the message increases from ~ 3.5h (without stress) to >10h (with stress) for proliferative cells and from ~5.5h (without stress) to >10h (with stress) for senescent cells (Figure 8D). Therefore, the observed stabilization of p21 mRNA under these conditions correlates with the formation of stress granules during senescence. Our data suggest that the recruitment of p21 mRNA to stress granules leads to decreased translation in senescent cells. Indeed, specific protein-mRNA interactions are required for the stabilization of mRNAs in stressed cells. A recent study has shown that ZBP1 (Zipcode-binding protein 1) stabilizes its target messages c-myc and β-actin in stress granules (Stöhr et al., 2006). Another RNA-stabilizing protein, HuR, was also shown to stabilize p21 mRNA under UV treatment (Wang et al., 2000). Recent study has demonstrated that HuR colocalizes with p21 mRNA in stress granules (Mazroui et al., 2007). However, there has been no direct evidence that HuR stabilizes mRNAs in stress granules. Therefore, it will be interesting to investigate further the proteins which stabilize p21 mRNA in stress granules.

CUGBP1, the RNA binding protein, regulates p21 translation during senescence (Stein et al., 1999). Therefore, it was possible that CUGBP1 could also be recruited to SGs in response to AS treatment which will explain in part the negative effect on the translation

of p21 mRNA. Our results confirm this possibility and show that while the pattern of CUGBP1 expression does not change significantly between unstressed and stressed cells during senescence (Figure 10), it is recruited to stress granules in response to AS treatment (Figure 10). Therefore, together our data show that recruitment of both p21 mRNA and CUGBP1 in stress granules leads to the considerable decrease in the expression of p21 protein in senescence cells during oxidative stress. Since the assembly of stress granules is reversible (Figure 5), p21 mRNA and CUGBP1 should be available for the recovery of p21 translation after removal of stress. Our data show that removal of stress leads to recovery of p21 translation in both early and late senescent cells (Figure 11), which correlates with the disassembly of stress granules. We have previously shown that the increased translation of p21 correlates with the disassembly of stress granules in HeLa cells during prolonged inhibition of ubiquitin-proteasome system (Mazroui et al., 2007). Our results show that in early senescent cells, p21 translation is increased during their recovery but fail to maintain the trend of recovery of p21 translation (Figure 11B) since unstressed cells have lower level of p21 than stressed cells in early senescence (Figure 7C).

In this thesis, we provide evidence that stress granules can be induced in senescent cells and the show that one of major consequence of their assembly is the rapid reduction in the translation of p21 mRNA due to the trapped mRNA in the granules. It will be of high interest to investigate further the molecular mechanisms responsible for the increased induction of stress granules in senescent cells. More importantly, our study paves a new avenue to learning how stress granules may mediate the response of senescent cells to environmental stresses and thus may help us understand how senescence contributes to late-life related cancers and other diseases.

REFERENCES

Adams, P.D. 2007. Remodeling chromatin for senescence. *Aging Cell.* 6:425-7.

Adler, V., L.R. Dolan, J. Kim, M. Pincus, J.C. Barrett, and Z. Ronai. 1996. Changes in jun N-terminal kinase activation by stress during aging of cultured normal human fibroblasts. *Mol Carcinog.* 17:8-12.

Aggarwal, S., and S. Gupta. 1998. Increased apoptosis of T cell subsets in aging humans: altered expression of Fas (CD95), Fas ligand, Bcl-2, and Bax. *J Immunol.* 160:1627-37.

Aggarwal, S., and S. Gupta. 1999. Increased activity of caspase 3 and caspase 8 in anti-Fas-induced apoptosis in lymphocytes from ageing humans. *Clin Exp Immunol.* 117:285-90.

Alway, S.E. 1997. Overload-induced C-Myc oncoprotein is reduced in aged skeletal muscle. *J Gerontol A Biol Sci Med Sci.* 52:B203-11.

Asghar, M., L. George, and M.F. Lokhandwala. 2007. Exercise decreases oxidative stress and inflammation and restores renal dopamine D1 receptor function in old rats. *Am J Physiol Renal Physiol.* 293:F914-9.

Balin, A.K., and L. Pratt. 2002. Oxygen modulates the growth of skin fibroblasts. *In Vitro Cell Dev Biol Anim.* 38:305-10.

Ballard, F.J., and L.C. Read. 1985. Changes in protein synthesis and breakdown rates and responsiveness to growth factors with ageing in human lung fibroblasts. *Mech Ageing Dev.* 30:11-22.

Bandyopadhyay, D., J.L. Curry, Q. Lin, H.W. Richards, D. Chen, P.J. Hornsby, N.A. Timchenko, and E.E. Medrano. 2007. Dynamic assembly of chromatin complexes during cellular senescence: implications for the growth arrest of human melanocytic nevi. *Aging Cell.* 6:577-91.

Beausejour, C.M., A. Krtolica, F. Galimi, M. Narita, S.W. Lowe, P. Yaswen, and J. Campisi. 2003. Reversal of human cellular senescence: roles of the p53 and p16 pathways. *Embo J.* 22:4212-22.

Ben-Porath, I., and R.A. Weinberg. 2005. The signals and pathways activating cellular senescence. *Int J Biochem Cell Biol.* 37:961-76.

Blake, M.J., R. Udelsman, G.J. Feulner, D.D. Norton, and N.J. Holbrook. 1991. Stress-induced heat shock protein 70 expression in adrenal cortex: an adrenocorticotropic hormone-sensitive, age-dependent response. *Proc Natl Acad Sci U S A.* 88:9873-7.

Blander, G., R.M. de Oliveira, C.M. Conboy, M. Haigis, and L. Guarente. 2003. Superoxide dismutase 1 knock-down induces senescence in human fibroblasts. *J Biol Chem.* 278:38966-9.

Blasco, M.A., H.W. Lee, M.P. Hande, E. Samper, P.M. Lansdorp, R.A. DePinho, and C.W. Greider. 1997. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell.* 91:25-34.

Borger, D.R., and J.A. DeCaprio. 2006. Targeting of p300/CREB binding protein coactivators by simian virus 40 is mediated through p53. *J Virol.* 80:4292-303.

Brenner, A.J., M.R. Stampfer, and C.M. Aldaz. 1998. Increased p16 expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with p16 inactivation. *Oncogene.* 17:199-205.

Brondello, J.M., J. Pouyssegur, and F.R. McKenzie. 1999. Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. *Science*. 286:2514-7.

Brown, E.J., and S.L. Schreiber. 1996. A signaling pathway to translational control. *Cell*. 86:517-20.

Brown, J.P., W. Wei, and J.M. Sedivy. 1997. Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. *Science*. 277:831-4.

Bruunsgaard, H., and B.K. Pedersen. 2000. Special feature for the Olympics: effects of exercise on the immune system: effects of exercise on the immune system in the elderly population. *Immunol Cell Biol*. 78:523-31.

Calabrese, V., C. Colombrita, R. Sultana, G. Scapagnini, M. Calvani, D.A. Butterfield, and A.M. Stella. 2006. Redox modulation of heat shock protein expression by acetylcarnitine in aging brain: relationship to antioxidant status and mitochondrial function. *Antioxid Redox Signal*. 8:404-16.

Campisi, J. 2005. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell*. 120:513-22.

Cande, C., N. Vahsen, D. Metivier, H. Tourriere, K. Chebli, C. Garrido, J. Tazi, and G. Kroemer. 2004. Regulation of cytoplasmic stress granules by apoptosis-inducing factor. *J Cell Sci*. 117:4461-8.

Ceddia, M.A., E.A. Price, C.K. Kohlmeier, J.K. Evans, Q. Lu, E. McAuley, and J.A. Woods. 1999. Differential leukocytosis and lymphocyte mitogenic response to acute maximal exercise in the young and old. *Med Sci Sports Exerc*. 31:829-36.

Chen, J.J., N. Brot, and H. Weissbach. 1980. RNA and protein synthesis in cultured human fibroblasts derived from donors of various ages. *Mech Ageing Dev*. 13:285-95.

Chen, Q., and B.N. Ames. 1994. Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells. *Proc Natl Acad Sci U S A*. 91:4130-4.

Chondrogianni, N., F.L. Stratford, I.P. Trougakos, B. Friguet, A.J. Rivett, and E.S. Gonos. 2003. Central role of the proteasome in senescence and survival of human fibroblasts: induction of a senescence-like phenotype upon its inhibition and resistance to stress upon its activation. *J Biol Chem*. 278:28026-37.

Choudhury, A.R., Z. Ju, M.W. Djojosubroto, A. Schienke, A. Lechel, S. Schaetzlein, H. Jiang, A. Stepczynska, C. Wang, J. Buer, H.W. Lee, T. von Zglinicki, A. Ganser, P. Schirmacher, H. Nakauchi, and K.L. Rudolph. 2007. Cdkn1a deletion improves stem cell function and lifespan of mice with dysfunctional telomeres without accelerating cancer formation. *Nat Genet*. 39:99-105.

Chung, L., and Y.C. Ng. 2006. Age-related alterations in expression of apoptosis regulatory proteins and heat shock proteins in rat skeletal muscle. *Biochim Biophys Acta*. 1762:103-9.

Collado, M., M.A. Blasco, and M. Serrano. 2007a. Cellular senescence in cancer and aging. *Cell*. 130:223-33.

Collado, M., M.A. Blasco, and M. Serrano. 2007b. Cellular senescence in cancer and aging. *Cell*. 130:223-33.

Coppe, J.P., K. Kauser, J. Campisi, and C.M. Beausejour. 2006. Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. *J Biol Chem*. 281:29568-74.

Cristofalo, V.J., A. Lorenzini, R.G. Allen, C. Torres, and M. Tresini. 2004. Replicative senescence: a critical review. *Mech Ageing Dev.* 125:827-48.

Csermely, P., I. Penzes, and S. Toth. 1995. Chronic overcrowding decreases cytoplasmic free calcium levels in T lymphocytes of aged CBA/CA mice. *Experientia.* 51:976-9.

d'Adda di Fagagna, F., P.M. Reaper, L. Clay-Farrace, H. Fiegler, P. Carr, T. Von Zglinicki, G. Saretzki, N.P. Carter, and S.P. Jackson. 2003. A DNA damage checkpoint response in telomere-initiated senescence. *Nature.* 426:194-8.

Dang, Y., N. Kedersha, W.K. Low, D. Romo, M. Gorospe, R. Kaufman, P. Anderson, and J.O. Liu. 2006. Eukaryotic initiation factor 2alpha-independent pathway of stress granule induction by the natural product pateamine A. *J Biol Chem.* 281:32870-8.

David, P.S., R. Tanveer, and J.D. Port. 2007. FRET-detectable interactions between the ARE binding proteins, HuR and p37AUF1. *Rna.* 13:1453-68.

De Blasi, A., M. Fratelli, M. Wielosz, and M. Lipartiti. 1987. Regulation of beta adrenergic receptors on rat mononuclear leukocytes by stress: receptor redistribution and down-regulation are altered with aging. *J Pharmacol Exp Ther.* 240:228-33.

De la Fuente, M., M.D. Ferrandez, M. Del Rio, M. Sol Burgos, and J. Miquel. 1998. Enhancement of leukocyte functions in aged mice supplemented with the antioxidant thioproline. *Mech Ageing Dev.* 104:213-25.

DeJesus, V., I. Rios, C. Davis, Y. Chen, D. Calhoun, Z. Zakeri, and K. Hubbard. 2002. Induction of apoptosis in human replicative senescent fibroblasts. *Exp Cell Res.* 274:92-9.

Deppert, W., M. Haug, and T. Steinmayer. 1987. Modulation of p53 protein expression during cellular transformation with simian virus 40. *Mol Cell Biol.* 7:4453-63.

Dimri, G.P., X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E.E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith, and et al. 1995a. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A.* 92:9363-7.

Dimri, G.P., X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E.E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith, and et al. 1995b. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A.* 92:9363-7.

Effros, R.B., X. Zhu, and R.L. Walford. 1994. Stress response of senescent T lymphocytes: reduced hsp70 is independent of the proliferative block. *J Gerontol.* 49:B65-70.

el-Deiry, W.S., J.W. Harper, P.M. O'Connor, V.E. Velculescu, C.E. Canman, J. Jackman, J.A. Pietenpol, M. Burrell, D.E. Hill, Y. Wang, and et al. 1994. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res.* 54:1169-74.

el-Deiry, W.S., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell.* 75:817-25.

Ellis, R.E., J.Y. Yuan, and H.R. Horvitz. 1991. Mechanisms and functions of cell death. *Annu Rev Cell Biol.* 7:663-98.

Emara, M.M., and M.A. Brinton. 2007. Interaction of TIA-1/TIAR with West Nile and dengue virus products in infected cells interferes with stress granule formation and processing body assembly. *Proc Natl Acad Sci U S A.* 104:9041-6.

Farges, M.C., F. Raul, J.P. Cezard, P. Davot, M.T. Meunier, L. Cynober, and M.P. Vasson. 1998. A pancreatic extract-enriched diet corrects ileal mucosa atrophy in endotoxemic aged rats. *Dig Dis Sci.* 43:2244-50.

Fonager, J., R. Beedholm, B.F. Clark, and S.I. Rattan. 2002. Mild stress-induced stimulation of heat-shock protein synthesis and improved functional ability of human fibroblasts undergoing aging in vitro. *Exp Gerontol.* 37:1223-8.

Gabai, V.L., and M.Y. Sherman. 2002. Invited review: Interplay between molecular chaperones and signaling pathways in survival of heat shock. *J Appl Physiol.* 92:1743-8.

Gallouzi, I.E., C.M. Brennan, and J.A. Steitz. 2001. Protein ligands mediate the CRM1-dependent export of HuR in response to heat shock. *Rna.* 7:1348-61.

Gallouzi, I.E., C.M. Brennan, M.G. Stenberg, M.S. Swanson, A. Eversole, N. Maizels, and J.A. Steitz. 2000a. HuR binding to cytoplasmic mRNA is perturbed by heat shock. *Proc Natl Acad Sci U S A.* 97:3073-8.

Gallouzi, I.E., C.M. Brennan, M.G. Stenberg, M.S. Swanson, A. Eversole, N. Maizels, and J.A. Steitz. 2000b. HuR binding to cytoplasmic mRNA is perturbed by heat shock. *Proc Natl Acad Sci U S A.* 97:3073-8.

Gebauer, F., and M.W. Hentze. 2004. Molecular mechanisms of translational control. *Nat Rev Mol Cell Biol.* 5:827-35.

Gil, J., and G. Peters. 2006. Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. *Nat Rev Mol Cell Biol.* 7:667-77.

Gilks, N., N. Kedersha, M. Ayodele, L. Shen, G. Stoecklin, L.M. Dember, and P. Anderson. 2004. Stress granule assembly is mediated by prion-like aggregation of TIA-1. *Mol Biol Cell.* 15:5383-98.

Ginaldi, L., M. De Martinis, A. D'Ostilio, L. Marini, M.F. Loreto, M.P. Corsi, and D. Quaglino. 2000. Cell proliferation and apoptosis in the immune system in the elderly. *Immunol Res.* 21:31-8.

Gingras, A.C., B. Raught, and N. Sonenberg. 1999. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem.* 68:913-63.

Green, D.R., and J.C. Reed. 1998. Mitochondria and apoptosis. *Science.* 281:1309-12.

Guayerbas, N., M. Puerto, M.D. Ferrandez, and M. De La Fuente. 2002. A diet supplemented with thiolic anti-oxidants improves leucocyte function in two strains of prematurely ageing mice. *Clin Exp Pharmacol Physiol.* 29:1009-14.

Gupta, S. 2000. Molecular and biochemical pathways of apoptosis in lymphocytes from aged humans. *Vaccine.* 18:1596-601.

Hahn, W.C., and R.A. Weinberg. 2002. Rules for making human tumor cells. *N Engl J Med.* 347:1593-603.

Hall, D.M., L. Xu, V.J. Drake, L.W. Oberley, T.D. Oberley, P.L. Moseley, and K.C. Kregel. 2000. Aging reduces adaptive capacity and stress protein expression in the liver after heat stress. *J Appl Physiol.* 89:749-59.

Hara, E., R. Smith, D. Parry, H. Tahara, S. Stone, and G. Peters. 1996. Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. *Mol Cell Biol.* 16:859-67.

Harman, D. 1956. Aging: a theory based on free radical and radiation chemistry. *J Gerontol.* 11:298-300.

Hata, R. 1990. Age-dependent changes in collagen metabolism and response to hypertonic culture conditions of rat aortic smooth muscle cells and skin fibroblasts. *Cell Biol Int Rep.* 14:25-33.

Hayflick, L. 1965. The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Exp Cell Res.* 37:614-36.

Hayflick, L. 1985. The cell biology of aging. *Clin Geriatr Med.* 1:15-27.

Hayflick, L. 1998. How and why we age. *Exp Gerontol.* 33:639-53.

Hazane, F., K. Valenti, S. Sauvaigo, A. Peinnequin, C. Mouret, A. Favier, and J.C. Beani. 2005. Ageing effects on the expression of cell defence genes after UVA irradiation in human male cutaneous fibroblasts using cDNA arrays. *J Photochem Photobiol B.* 79:171-90.

Helenius, M., L. Makelainen, and A. Salminen. 1999. Attenuation of NF-kappaB signaling response to UVB light during cellular senescence. *Exp Cell Res.* 248:194-202.

Hipkiss, A.R. 2006. On the mechanisms of ageing suppression by dietary restriction-is persistent glycolysis the problem? *Mech Ageing Dev.* 127:8-15.

Holcik, M., and N. Sonenberg. 2005. Translational control in stress and apoptosis. *Nat Rev Mol Cell Biol.* 6:318-27.

Honda, S., and M. Matsuo. 1987. Lack of recovery from oxygen-induced damage to colony formation and DNA synthesis in senescent human diploid fibroblasts. *Mech Ageing Dev.* 40:81-7.

Horohov, D.W., A. Dimock, P. Guirnalda, R.W. Folsom, K.H. McKeever, and K. Malinowski. 1999. Effect of exercise on the immune response of young and old horses. *Am J Vet Res.* 60:643-7.

Hsieh, J.K., D. Kletsas, G. Clunn, A.D. Hughes, M. Schachter, and C. Demoliou-Mason. 2000. p53, p21(WAF1/CIP1), and MDM2 involvement in the proliferation and apoptosis in an in vitro model of conditionally immortalized human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 20:973-81.

Hua, Y., and J. Zhou. 2004. Survival motor neuron protein facilitates assembly of stress granules. *FEBS Lett.* 572:69-74.

Hussain, S.G., and K.V. Ramaiah. 2007. Reduced eIF2alpha phosphorylation and increased proapoptotic proteins in aging. *Biochem Biophys Res Commun.* 355:365-70.

Iakova, P., G.L. Wang, L. Timchenko, M. Michalak, O.M. Pereira-Smith, J.R. Smith, and N.A. Timchenko. 2004a. Competition of CUGBP1 and calreticulin for the regulation of p21 translation determines cell fate. *Embo J.* 23:406-17.

Iakova, P., G.L. Wang, L. Timchenko, M. Michalak, O.M. Pereira-Smith, J.R. Smith, and N.A. Timchenko. 2004b. Competition of CUGBP1 and calreticulin for the regulation of p21 translation determines cell fate. *Embo J.* 23:406-17.

Isoyama, S. 1996. Age-related changes before and after imposition of hemodynamic stress in the mammalian heart. *Life Sci.* 58:1601-14.

Iwasa, H., J. Han, and F. Ishikawa. 2003. Mitogen-activated protein kinase p38 defines the common senescence-signalling pathway. *Genes Cells.* 8:131-44.

Joaquin, A.M., and S. Gollapudi. 2001. Functional decline in aging and disease: a role for apoptosis. *J Am Geriatr Soc.* 49:1234-40.

Ju, Z., A.R. Choudhury, and K.L. Rudolph. 2007. A dual role of p21 in stem cell aging. *Ann N Y Acad Sci.* 1100:333-44.

Kang, H., K. Cui, and K. Zhao. 2004. BRG1 controls the activity of the retinoblastoma protein via regulation of p21CIP1/WAF1/SDI. *Mol Cell Biol.* 24:1188-99.

Karlseder, J., A. Smogorzewska, and T. de Lange. 2002. Senescence induced by altered telomere state, not telomere loss. *Science.* 295:2446-9.

Kedersha, N., and P. Anderson. 2002. Stress granules: sites of mRNA triage that regulate mRNA stability and translatability. *Biochem Soc Trans.* 30:963-9.

Kedersha, N., M.R. Cho, W. Li, P.W. Yacono, S. Chen, N. Gilks, D.E. Golan, and P. Anderson. 2000. Dynamic shuttling of TIA-1 accompanies the recruitment of mRNA to mammalian stress granules. *J Cell Biol.* 151:1257-68.

Kedersha, N., G. Stoecklin, M. Ayodele, P. Yacono, J. Lykke-Andersen, M.J. Fitzler, D. Scheuner, R.J. Kaufman, D.E. Golan, and P. Anderson. 2005a. Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *J Cell Biol.* 169:871-84.

Kedersha, N., G. Stoecklin, M. Ayodele, P. Yacono, J. Lykke-Andersen, M.J. Fritzler, D. Scheuner, R.J. Kaufman, D.E. Golan, and P. Anderson. 2005b. Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *J Cell Biol.* 169:871-84.

Kedersha, N.L., M. Gupta, W. Li, I. Miller, and P. Anderson. 1999a. RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules. *J Cell Biol.* 147:1431-42.

Kedersha, N.L., M. Gupta, W. Li, I. Miller, and P. Anderson. 1999b. RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules. *J Cell Biol.* 147:1431-42.

Khan, S., C. Guevara, G. Fujii, and D. Parry. 2004. p14ARF is a component of the p53 response following ionizing irradiation of normal human fibroblasts. *Oncogene.* 23:6040-6.

Kim, K., K. Nose, and M. Shibanuma. 2000. Significance of nuclear relocalization of ERK1/2 in reactivation of c-fos transcription and DNA synthesis in senescent fibroblasts. *J Biol Chem.* 275:20685-92.

Kim, W.J., S.H. Back, V. Kim, I. Ryu, and S.K. Jang. 2005. Sequestration of TRAF2 into stress granules interrupts tumor necrosis factor signaling under stress conditions. *Mol Cell Biol.* 25:2450-62.

Kim, W.Y., and N.E. Sharpless. 2006. The regulation of INK4/ARF in cancer and aging. *Cell.* 127:265-75.

Kimball, S.R., R.L. Horetsky, D. Ron, L.S. Jefferson, and H.P. Harding. 2003. Mammalian stress granules represent sites of accumulation of stalled translation initiation complexes. *Am J Physiol Cell Physiol.* 284:C273-84.

Klein-Nulend, J., J.G. Sterck, C.M. Semeins, P. Lips, M. Joldersma, J.A. Baart, and E.H. Burger. 2002. Donor age and mechanosensitivity of human bone cells. *Osteoporos Int.* 13:137-46.

Kortlever, R.M., P.J. Higgins, and R. Bernards. 2006. Plasminogen activator inhibitor-1 is a critical downstream target of p53 in the induction of replicative senescence. *Nat Cell Biol.* 8:877-84.

Kraft, D.C., C.C. Deocaris, R. Wadhwa, and S.I. Rattan. 2006. Preincubation with the proteasome inhibitor MG-132 enhances proteasome activity via the Nrf2 transcription factor in aging human skin fibroblasts. *Ann N Y Acad Sci.* 1067:420-4.

Kregel, K.C., and P.L. Moseley. 1996. Differential effects of exercise and heat stress on liver HSP70 accumulation with aging. *J Appl Physiol.* 80:547-51.

Kregel, K.C., P.L. Moseley, R. Skidmore, J.A. Gutierrez, and V. Guerriero, Jr. 1995. HSP70 accumulation in tissues of heat-stressed rats is blunted with advancing age. *J Appl Physiol.* 79:1673-8.

Krtolica, A., and J. Campisi. 2002. Cancer and aging: a model for the cancer promoting effects of the aging stroma. *Int J Biochem Cell Biol.* 34:1401-14.

Krtolica, A., S. Parrinello, S. Lockett, P.Y. Desprez, and J. Campisi. 2001. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci U S A.* 98:12072-7.

Kumaran, S., B. Deepak, B. Naveen, and C. Panneerselvam. 2003. Effects of levocarnitine on mitochondrial antioxidant systems and oxidative stress in aged rats. *Drugs R D.* 4:141-7.

Lauc, G., A.P. Seve, J. Hubert, M. Flogel-Mrsic, W.E. Muller, and H.C. Schroder. 1993. HnRNP CBP35.CBP67 interaction during stress response and ageing. *Mech Ageing Dev.* 70:227-36.

Lee, Y.K., D. Manalo, and A.Y. Liu. 1996. Heat shock response, heat shock transcription factor and cell aging. *Biol Signals.* 5:180-91.

Liu, R.Y., X. Li, L. Li, and G.C. Li. 1992. Expression of human hsp70 in rat fibroblasts enhances cell survival and facilitates recovery from translational and transcriptional inhibition following heat shock. *Cancer Res.* 52:3667-73.

Luce, M.C., and V.J. Cristofalo. 1992. Reduction in heat shock gene expression correlates with increased thermosensitivity in senescent human fibroblasts. *Exp Cell Res.* 202:9-16.

Macieira-Coelho, A., and L. Lima. 1973. Aging in vitro: incorporation of RNA and protein precursors and acid phosphatase activity during the lifespan of chick embryo fibroblasts. *Mech Ageing Dev.* 2:13-8.

Makrides, S.C. 1983. Protein synthesis and degradation during aging and senescence. *Biol Rev Camb Philos Soc.* 58:343-422.

Maldonado, J.L., L. Timmerman, J. Fridlyand, and B.C. Bastian. 2004. Mechanisms of cell-cycle arrest in Spitz nevi with constitutive activation of the MAP-kinase pathway. *Am J Pathol.* 164:1783-7.

Marcotte, R., C. Lacelle, and E. Wang. 2004. Senescent fibroblasts resist apoptosis by downregulating caspase-3. *Mech Ageing Dev.* 125:777-83.

Marignani, P.A., F. Kanai, and C.L. Carpenter. 2001. LKB1 associates with Brg1 and is necessary for Brg1-induced growth arrest. *J Biol Chem.* 276:32415-8.

Mawal-Dewan, M., L. Frisoni, V.J. Cristofalo, and C. Sell. 2003. Extension of replicative lifespan in WI-38 human fibroblasts by dexamethasone treatment is accompanied by suppression of p21 Waf1/Cip1/Sdi1 levels. *Exp Cell Res.* 285:91-8.

Mazan-Mamczarz, K., A. Lal, J.L. Martindale, T. Kawai, and M. Gorospe. 2006. Translational repression by RNA-binding protein TIAR. *Mol Cell Biol.* 26:2716-27.

Mazroui, R., S. Di Marco, R.J. Kaufman, and I.E. Gallouzi. 2007a. Inhibition of the ubiquitin-proteasome system induces stress granule formation. *Mol Biol Cell.* 18:2603-18.

Mazroui, R., S. Di Marco, R.J. Kaufman, and I.E. Gallouzi. 2007b. Inhibition of the ubiquitin-proteasome system induces stress granule formation. *Mol Biol Cell.* 18:2603-18.

Mazroui, R., M.E. Huot, S. Tremblay, C. Filion, Y. Labelle, and E.W. Khandjian. 2002a. Trapping of messenger RNA by Fragile X Mental Retardation protein into cytoplasmic granules induces translation repression. *Hum Mol Genet.* 11:3007-17.

Mazroui, R., M.E. Huot, S. Tremblay, C. Filion, Y. Labelle, and E.W. Khandjian. 2002b. Trapping of messenger RNA by Fragile X Mental Retardation protein into cytoplasmic granules induces translation repression. *Hum Mol Genet.* 11:3007-17.

Mazroui, R., R. Sukarieh, M.E. Bordeleau, R.J. Kaufman, P. Northcote, J. Tanaka, I. Gallouzi, and J. Pelletier. 2006. Inhibition of ribosome recruitment induces stress granule formation independently of eukaryotic initiation factor 2alpha phosphorylation. *Mol Biol Cell.* 17:4212-9.

Merker, K., N. Sitte, and T. Grune. 2000. Hydrogen peroxide-mediated protein oxidation in young and old human MRC-5 fibroblasts. *Arch Biochem Biophys.* 375:50-4.

Minamino, T., S.A. Mitsialis, and S. Kourembanas. 2001. Hypoxia extends the life span of vascular smooth muscle cells through telomerase activation. *Mol Cell Biol.* 21:3336-42.

Miura, S., M. Yamaguchi, N. Shimizu, and Y. Abiko. 2000. Mechanical stress enhances expression and production of plasminogen activator in aging human periodontal ligament cells. *Mech Ageing Dev.* 112:217-31.

Miura, Y., M. Kano, K. Abe, S. Urano, S. Suzuki, and T. Toda. 2005. Age-dependent variations of cell response to oxidative stress: proteomic approach to protein expression and phosphorylation. *Electrophoresis.* 26:2786-96.

Naito, H., S.K. Powers, H.A. Demirel, and J. Aoki. 2001. Exercise training increases heat shock protein in skeletal muscles of old rats. *Med Sci Sports Exerc.* 33:729-34.

Nakayama, J., H. Tahara, E. Tahara, M. Saito, K. Ito, H. Nakamura, T. Nakanishi, E. Tahara, T. Ide, and F. Ishikawa. 1998. Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. *Nat Genet.* 18:65-8.

Napolitano, M.A., M. Cipollaro, A. Cascino, M.A. Melone, A. Giordano, and U. Galderisi. 2007. Brg1 chromatin remodeling factor is involved in cell growth arrest, apoptosis and senescence of rat mesenchymal stem cells. *J Cell Sci.* 120:2904-11.

Nielsen, G.P., A.O. Stemmer-Rachamimov, J. Shaw, J.E. Roy, J. Koh, and D.N. Louis. 1999. Immunohistochemical survey of p16INK4A expression in normal human adult and infant tissues. *Lab Invest.* 79:1137-43.

Nishimura, E.K., S.R. Granter, and D.E. Fisher. 2005. Mechanisms of hair graying: incomplete melanocyte stem cell maintenance in the niche. *Science.* 307:720-4.

Ogami, M., Y. Ikura, M. Ohsawa, T. Matsuo, S. Kayo, N. Yoshimi, E. Hai, N. Shirai, S. Ehara, R. Komatsu, T. Naruko, and M. Ueda. 2004. Telomere shortening in human coronary artery diseases. *Arterioscler Thromb Vasc Biol.* 24:546-50.

Ohshima, S. 2004. Apoptosis in stress-induced and spontaneously senescent human fibroblasts. *Biochem Biophys Res Commun.* 324:241-6.

Ohshima, S. 2006. Apoptosis and necrosis in senescent human fibroblasts. *Ann N Y Acad Sci.* 1067:228-34.

Ohtani, N., K. Yamakoshi, A. Takahashi, and E. Hara. 2004. The p16INK4a-RB pathway: molecular link between cellular senescence and tumor suppression. *J Med Invest.* 51:146-53.

Ohzeki, K., M. Yamaguchi, N. Shimizu, and Y. Abiko. 1999. Effect of cellular aging on the induction of cyclooxygenase-2 by mechanical stress in human periodontal ligament cells. *Mech Ageing Dev.* 108:151-63.

Oruetxebarria, I., F. Venturini, T. Kekarainen, A. Houweling, L.M. Zuijderduijn, A. Mohd-Sarip, R.G. Vries, R.C. Hoeben, and C.P. Verrijzer. 2004. P16INK4a is required for hSNF5 chromatin remodeler-induced cellular senescence in malignant rhabdoid tumor cells. *J Biol Chem.* 279:3807-16.

Packer, L., and K. Fuehr. 1977. Low oxygen concentration extends the lifespan of cultured human diploid cells. *Nature.* 267:423-5.

Padgett, D.A., R.C. MacCallum, and J.F. Sheridan. 1998. Stress exacerbates age-related decrements in the immune response to an experimental influenza viral infection. *J Gerontol A Biol Sci Med Sci.* 53:B347-53.

Pantoja, C., and M. Serrano. 1999. Murine fibroblasts lacking p21 undergo senescence and are resistant to transformation by oncogenic Ras. *Oncogene.* 18:4974-82.

Pardue, S., K. Groshan, J.D. Raese, and M. Morrison-Bogorad. 1992. Hsp70 mRNA induction is reduced in neurons of aged rat hippocampus after thermal stress. *Neurobiol Aging.* 13:661-72.

Parrinello, S., J.P. Coppe, A. Krtolica, and J. Campisi. 2005. Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. *J Cell Sci.* 118:485-96.

Parrinello, S., E. Samper, A. Krtolica, J. Goldstein, S. Melov, and J. Campisi. 2003. Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nat Cell Biol.* 5:741-7.

Petropoulos, I., M. Conconi, X. Wang, B. Hoenel, F. Bregegere, Y. Milner, and B. Friguet. 2000. Increase of oxidatively modified protein is associated with a decrease of proteasome activity and content in aging epidermal cells. *J Gerontol A Biol Sci Med Sci.* 55:B220-7.

Pullen, N., P.B. Dennis, M. Andjelkovic, A. Dufner, S.C. Kozma, B.A. Hemmings, and G. Thomas. 1998. Phosphorylation and activation of p70s6k by PDK1. *Science.* 279:707-10.

Raaben, M., M.J. Groot Koerkamp, P.J. Rottier, and C.A. de Haan. 2007. Mouse hepatitis coronavirus replication induces host translational shutoff and mRNA decay, with concomitant formation of stress granules and processing bodies. *Cell Microbiol.* 9:2218-29.

Rattan, S.I. 1998. Repeated mild heat shock delays ageing in cultured human skin fibroblasts. *Biochem Mol Biol Int.* 45:753-9.

Ressler, S., J. Bartkova, H. Niederegger, J. Bartek, K. Scharffetter-Kochanek, P. Jansen-Durr, and M. Wlaschek. 2006. p16INK4A is a robust in vivo biomarker of cellular aging in human skin. *Aging Cell.* 5:379-89.

Riabowol, K.T., L.A. Mizzen, and W.J. Welch. 1988. Heat shock is lethal to fibroblasts microinjected with antibodies against hsp70. *Science.* 242:433-6.

Rohrbach, S., S. Gruenler, M. Teschner, and J. Holtz. 2006. The thioredoxin system in aging muscle: key role of mitochondrial thioredoxin reductase in the protective

effects of caloric restriction? *Am J Physiol Regul Integr Comp Physiol.* 291:R927-35.

Romani, R.J., I.K. Yu, L.L. Ku, L.K. Fisher, and N. Dehgan. 1968. Cellular Senescence, Radiation Damage to Mitochondria, and the Compensatory Response in Ripening Pear Fruits. *Plant Physiol.* 43:1089-1096.

Rosenfeldt, F., F. Miller, P. Nagley, A. Hadj, S. Marasco, D. Quick, F. Sheeran, M. Wowk, and S. Pepe. 2004. Response of the senescent heart to stress: clinical therapeutic strategies and quest for mitochondrial predictors of biological age. *Ann N Y Acad Sci.* 1019:78-84.

Rowland, M.A., P. Nagley, A.W. Linnane, and F.L. Rosenfeldt. 1998. Coenzyme Q10 treatment improves the tolerance of the senescent myocardium to pacing stress in the rat. *Cardiovasc Res.* 40:165-73.

Rylander, M.N., K.R. Diller, S. Wang, and S.J. Aggarwal. 2005. Correlation of HSP70 expression and cell viability following thermal stimulation of bovine aortic endothelial cells. *J Biomech Eng.* 127:751-7.

Ryu, S.J., K.A. Cho, Y.S. Oh, and S.C. Park. 2006. Role of Src-specific phosphorylation site on focal adhesion kinase for senescence-associated apoptosis resistance. *Apoptosis.* 11:303-13.

Ryu, S.J., Y.S. Oh, and S.C. Park. 2007. Failure of stress-induced downregulation of Bcl-2 contributes to apoptosis resistance in senescent human diploid fibroblasts. *Cell Death Differ.* 14:1020-8.

Sapolsky, R.M., and T.M. Donnelly. 1985. Vulnerability to stress-induced tumor growth increases with age in rats: role of glucocorticoids. *Endocrinology.* 117:662-6.

Satyanarayana, A., S.U. Wiemann, J. Buer, J. Lauber, K.E. Dittmar, T. Wustefeld, M.A. Blasco, M.P. Manns, and K.L. Rudolph. 2003. Telomere shortening impairs organ regeneration by inhibiting cell cycle re-entry of a subpopulation of cells. *Embo J.* 22:4003-13.

Sayama, K., Y. Shirakata, K. Midorikawa, Y. Hanakawa, and K. Hashimoto. 1999. Possible involvement of p21 but not of p16 or p53 in keratinocyte senescence. *J Cell Physiol.* 179:40-4.

Seluanov, A., V. Gorbunova, A. Falcovitz, A. Sigal, M. Milyavsky, I. Zurer, G. Shohat, N. Goldfinger, and V. Rotter. 2001. Change of the death pathway in senescent human fibroblasts in response to DNA damage is caused by an inability to stabilize p53. *Mol Cell Biol.* 21:1552-64.

Seo, A.Y., T. Hofer, B. Sung, S. Judge, H.Y. Chung, and C. Leeuwenburgh. 2006. Hepatic oxidative stress during aging: effects of 8% long-term calorie restriction and lifelong exercise. *Antioxid Redox Signal.* 8:529-38.

Serrano, M., and M.A. Blasco. 2001a. Putting the stress on senescence. *Curr Opin Cell Biol.* 13:748-53.

Serrano, M., and M.A. Blasco. 2001b. Putting the stress on senescence. *Curr Opin Cell Biol.* 13:748-53.

Serrano, M., H. Lee, L. Chin, C. Cordon-Cardo, D. Beach, and R.A. DePinho. 1996. Role of the INK4a locus in tumor suppression and cell mortality. *Cell.* 85:27-37.

Serrano, M., A.W. Lin, M.E. McCurrach, D. Beach, and S.W. Lowe. 1997a. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell.* 88:593-602.

Serrano, M., A.W. Lin, M.E. McCurrach, D. Beach, and S.W. Lowe. 1997b. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell.* 88:593-602.

Servais, S., A. Boussouar, A. Molnar, T. Douki, J.M. Pequignot, and R. Favier. 2005. Age-related sensitivity to lung oxidative stress during ozone exposure. *Free Radic Res.* 39:305-16.

Shakespeare, V., and J.H. Buchanan. 1976. Increased degradation rates of protein in aging human fibroblasts and in cells treated with an amino acid analog. *Exp Cell Res.* 100:1-8.

Shanahan, F., W. Seghezzi, D. Parry, D. Mahony, and E. Lees. 1999. Cyclin E associates with BAF155 and BRG1, components of the mammalian SWI-SNF complex, and alters the ability of BRG1 to induce growth arrest. *Mol Cell Biol.* 19:1460-9.

Shapiro, G.I., C.D. Edwards, M.E. Ewen, and B.J. Rollins. 1998a. p16INK4A participates in a G1 arrest checkpoint in response to DNA damage. *Mol Cell Biol.* 18:378-87.

Shapiro, G.I., C.D. Edwards, M.E. Ewen, and B.J. Rollins. 1998b. p16INK4A participates in a G1 arrest checkpoint in response to DNA damage. *Mol Cell Biol.* 18:378-87.

Shapiro, G.I., C.D. Edwards, and B.J. Rollins. 2000. The physiology of p16(INK4A)-mediated G1 proliferative arrest. *Cell Biochem Biophys.* 33:189-97.

Shapiro, G.I., J.E. Park, C.D. Edwards, L. Mao, A. Merlo, D. Sidransky, M.E. Ewen, and B.J. Rollins. 1995. Multiple mechanisms of p16INK4A inactivation in non-small cell lung cancer cell lines. *Cancer Res.* 55:6200-9.

Shida, M., and S. Isoyama. 1993. Effects of age on c-fos and c-myc gene expression in response to hemodynamic stress in isolated, perfused rat hearts. *J Mol Cell Cardiol.* 25:1025-35.

Shimizu, N., M. Yamaguchi, K. Uesu, T. Goseki, and Y. Abiko. 2000. Stimulation of prostaglandin E2 and interleukin-1beta production from old rat periodontal ligament cells subjected to mechanical stress. *J Gerontol A Biol Sci Med Sci.* 55:B489-95.

Siegl-Cachedenier, I., P. Munoz, J.M. Flores, P. Klatt, and M.A. Blasco. 2007. Deficient mismatch repair improves organismal fitness and survival of mice with dysfunctional telomeres. *Genes Dev.* 21:2234-47.

Simpson, R.J., G.D. Florida-James, C. Cosgrove, G.P. Whyte, S. Macrae, H. Pircher, and K. Guy. 2007. High-intensity exercise elicits the mobilization of senescent T lymphocytes into the peripheral blood compartment in human subjects. *J Appl Physiol.* 103:396-401.

Sitte, N., K. Merker, T. von Zglinicki, and T. Grune. 2000. Protein oxidation and degradation during proliferative senescence of human MRC-5 fibroblasts. *Free Radic Biol Med.* 28:701-8.

Siu, P.M., and S.E. Alway. 2005. Mitochondria-associated apoptotic signalling in denervated rat skeletal muscle. *J Physiol.* 565:309-23.

Smogorzewska, A., and T. de Lange. 2002. Different telomere damage signaling pathways in human and mouse cells. *Embo J.* 21:4338-48.

Solomon, S., Y. Xu, B. Wang, M.D. David, P. Schubert, D. Kennedy, and J.W. Schrader. 2007. Distinct structural features of caprin-1 mediate its interaction with G3BP-1 and its induction of phosphorylation of eukaryotic translation initiation factor 2alpha, entry to cytoplasmic stress granules, and selective interaction with a subset of mRNAs. *Mol Cell Biol.* 27:2324-42.

Starke-Reed, P.E., and C.N. Oliver. 1989. Protein oxidation and proteolysis during aging and oxidative stress. *Arch Biochem Biophys*. 275:559-67.

Stein, G.H., L.F. Drullinger, A. Soulard, and V. Dulic. 1999. Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. *Mol Cell Biol*. 19:2109-17.

Stephens, L., K. Anderson, D. Stokoe, H. Erdjument-Bromage, G.F. Painter, A.B. Holmes, P.R. Gaffney, C.B. Reese, F. McCormick, P. Tempst, J. Coadwell, and P.T. Hawkins. 1998. Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science*. 279:710-4.

Stoecklin, G., T. Stubbs, N. Kedersha, S. Wax, W.F. Rigby, T.K. Blackwell, and P. Anderson. 2004. MK2-induced tristetraprolin:14-3-3 complexes prevent stress granule association and ARE-mRNA decay. *Embo J*. 23:1313-24.

Stohr, N., M. Lederer, C. Reinke, S. Meyer, M. Hatzfeld, R.H. Singer, and S. Huttelmaier. 2006. ZBP1 regulates mRNA stability during cellular stress. *J Cell Biol*. 175:527-34.

Tavernarakis, N. 2007. Protein synthesis and aging: eIF4E and the soma vs. germline distinction. *Cell Cycle*. 6:1168-71.

te Poele, R.H., A.L. Okorokov, L. Jardine, J. Cummings, and S.P. Joel. 2002. DNA damage is able to induce senescence in tumor cells in vitro and in vivo. *Cancer Res*. 62:1876-83.

Toker, A., and L.C. Cantley. 1997. Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature*. 387:673-6.

Torres, C., M.K. Francis, A. Lorenzini, M. Tresini, and V.J. Cristofalo. 2003. Metabolic stabilization of MAP kinase phosphatase-2 in senescence of human fibroblasts. *Exp Cell Res*. 290:195-206.

Tourriere, H., K. Chebli, L. Zekri, B. Courselaud, J.M. Blanchard, E. Bertrand, and J. Tazi. 2003a. The RasGAP-associated endoribonuclease G3BP assembles stress granules. *J Cell Biol*. 160:823-31.

Tourriere, H., K. Chebli, L. Zekri, B. Courselaud, J.M. Blanchard, E. Bertrand, and J. Tazi. 2003b. The RasGAP-associated endoribonuclease G3BP assembles stress granules. *J Cell Biol*. 160:823-31.

Tuveson, D.A., A.T. Shaw, N.A. Willis, D.P. Silver, E.L. Jackson, S. Chang, K.L. Mercer, R. Grochow, H. Hock, D. Crowley, S.R. Hingorani, T. Zaks, C. King, M.A. Jacobetz, L. Wang, R.T. Bronson, S.H. Orkin, R.A. DePinho, and T. Jacks. 2004. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer Cell*. 5:375-87.

van Heemst, D., P.M. den Reijer, and R.G. Westendorp. 2007. Ageing or cancer: a review on the role of caretakers and gatekeepers. *Eur J Cancer*. 43:2144-52.

Van Nieuwenhoven, F.A., X. Martin, V.V. Heijnen, R.N. Cornelussen, and L.H. Snoeckx. 2001. HSP70-mediated acceleration of translational recovery after stress is independent of ribosomal RNA synthesis. *Eur J Cell Biol*. 80:586-92.

Vaziri, H., and S. Benchimol. 1998. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr Biol*. 8:279-82.

Verbeke, P., B.F. Clark, and S.I. Rattan. 2001. Reduced levels of oxidized and glycoxidized proteins in human fibroblasts exposed to repeated mild heat shock during serial passaging in vitro. *Free Radic Biol Med*. 31:1593-602.

von Zglinicki, T., and C.M. Martin-Ruiz. 2005. Telomeres as biomarkers for ageing and age-related diseases. *Curr Mol Med.* 5:197-203.

von Zglinicki, T., G. Saretzki, W. Docke, and C. Lotze. 1995. Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence? *Exp Cell Res.* 220:186-93.

Wakikawa, A., M. Utsuyama, A. Wakabayashi, M. Kitagawa, and K. Hirokawa. 1999. Vitamin E enhances the immune functions of young but not old mice under restraint stress. *Exp Gerontol.* 34:853-62.

Wang, E. 1995. Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. *Cancer Res.* 55:2284-92.

Wang, W., J.X. Chen, R. Liao, Q. Deng, J.J. Zhou, S. Huang, and P. Sun. 2002. Sequential activation of the MEK-extracellular signal-regulated kinase and MKK3/6-p38 mitogen-activated protein kinase pathways mediates oncogenic ras-induced premature senescence. *Mol Cell Biol.* 22:3389-403.

Wang, W., H. Furneaux, H. Cheng, M.C. Caldwell, D. Hutter, Y. Liu, N. Holbrook, and M. Gorospe. 2000. HuR regulates p21 mRNA stabilization by UV light. *Mol Cell Biol.* 20:760-9.

Wang, W., X. Yang, V.J. Cristofalo, N.J. Holbrook, and M. Gorospe. 2001a. Loss of HuR is linked to reduced expression of proliferative genes during replicative senescence. *Mol Cell Biol.* 21:5889-98.

Wang, W., X. Yang, V.J. Cristofalo, N.J. Holbrook, and M. Gorospe. 2001b. Loss of HuR is linked to reduced expression of proliferative genes during replicative senescence. *Mol Cell Biol.* 21:5889-98.

Wang, X., A. Flynn, A.J. Waskiewicz, B.L. Webb, R.G. Vries, I.A. Baines, J.A. Cooper, and C.G. Proud. 1998. The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways. *J Biol Chem.* 273:9373-7.

Wright, W.E., O.M. Pereira-Smith, and J.W. Shay. 1989. Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. *Mol Cell Biol.* 9:3088-92.

Wright, W.E., and J.W. Shay. 2000. Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology. *Nat Med.* 6:849-51.

Wu, B., M.J. Gu, A.R. Heydari, and A. Richardson. 1993. The effect of age on the synthesis of two heat shock proteins in the hsp70 family. *J Gerontol.* 48:B50-6.

Yeo, E.J., Y.C. Hwang, C.M. Kang, H.E. Choy, and S.C. Park. 2000. Reduction of UV-induced cell death in the human senescent fibroblasts. *Mol Cells.* 10:415-22.

Zarzhevsky, N., O. Menashe, E. Carmeli, H. Stein, and A.Z. Reznick. 2001. Capacity for recovery and possible mechanisms in immobilization atrophy of young and old animals. *Ann N Y Acad Sci.* 928:212-25.

Zhang, H., H. Hoff, T. Marinucci, V.J. Cristofalo, and C. Sell. 2000. Mitogen-independent phosphorylation of S6K1 and decreased ribosomal S6 phosphorylation in senescent human fibroblasts. *Exp Cell Res.* 259:284-92.

Zhu, J., D. Woods, M. McMahon, and J.M. Bishop. 1998. Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev.* 12:2997-3007.

Zindy, F., D.E. Quelle, M.F. Roussel, and C.J. Sherr. 1997. Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene*. 15:203-11.